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A Molecular Study of the Bacterioneuston and
its Role in the Air-Sea Exchange of Trace Gases

A thesis submitted by

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to

The Department of Biological Sciences

in fulfilment of the requirements for the degree of

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Abbreviations

μg	microgramme
μl	microlitre
μM	micromolar
16S rDNA	16S ribosomal RNA gene
\AA	Angstroms
ANMS	ammonium nitrate mineral salts
ASW	artificial sea water
atm	atmosphere
ATP	adenosine triphosphate
B_E	Biological gas exchange enhancement factor
BLAST	basic local alignment search tool
bp	base pairs
β	Bunsen solubility coefficient (v/v.atm^{-1})
CH_4	methane
cm	centimetre
C_w	concentration of a gas in water (M.l^{-1})
Da	Dalton
DGGE	denaturing gradient gel electrophoresis
DH_2O	distilled water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ECD	electron capture detector
EDTA	ethylenediaminetetraacetic acid (disodium salt)

EF	enrichment factor
EtBr	ethidium bromide
Ev	evasion
F	Gas flux
FID	flame ionisation detector
g	gramme
GC	gas chromatography
GWP	global warming potential
hr	hour
i.d.	internal diameter (mm)
Inv	invasive
k	total gas transfer velocity (cm.hr^{-1})
k_a	gas transfer velocity relative to air side measurements of gas concentration (cm.hr^{-1})
kb	kilobase
kDa	kiloDalton
KEF	kinetic energy flux
km	kilometre
k_w	gas transfer velocity relative to water side measurements of gas concentration (cm.hr^{-1})
l	litre
M	Molar
mg	milligramme
min	minute
ml	millilitre

mM	millimolar
MMO	methane monooxygenase
Mol	mole
MR	mixing ratio
n	number
<i>n</i>	Schmidt number exponent
N ₂ O	nitrous oxide
NAD	nicotinamide adenine dinucleotide (oxidised form)
NADH	nicotinamide adenine dinucleotide (reduced form)
ng	nanogramme
nm	nanometer
NMS	nitrate mineral salts
o/n	over night
°C	degrees centigrade
OD	optical density
ORF	open reading frame
OTU	operational taxonomic unit
<i>P</i>	ambient laboratory pressure
<i>P_a</i>	air density
PCR	polymerase chain reaction
<i>P_{ge}</i>	theoretical partial pressure after a contained water and air phase have reached chemical equilibrium
<i>pp</i>	partial pressure
ppbv	parts per billion (10 ⁻⁹) by volume
ppm	parts per million

ppmv	parts per million (10^{-6}) by volume
pptv	parts per trillion (10^{-12}) by volume
PQQ	pyrrolo-quinoline
P_w	water density
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal RNA
RuMP	ribulose monophosphate
s	second
<i>Sc</i>	Schmidt number
SDS	sodium dodecyl sulphate
SDS-page	SDS polyacrylamide gel electrophoresis
SF ₆	Sulfur hexafluoride
sp.	species
SSC	standard sodium citrate
TAE	tris-acetate-EDTA
Taq	<i>Thermus aquaticus</i>
TBE	tris-borate-EDTA
TE	tris-EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
Tg	terragramme (10^{12} g)
Tris	tris-(hydroxymethyl)-methylamine
UV	Ultraviolet
V	valve

v/v volume per volume

w/v weight per volume

Abstract

The two-way transfer of gases between oceans and air exerts an important control on the atmospheric inventories of climatically active gases such as CO₂, CH₄ and N₂O. Numerous physical variables are important to gas transfer at the sea surface and have complex interactions. Because of this, our current knowledge of the gas exchange process remains somewhat limited. Such a situation is urgently in need of attention if the effects of global change into the 21st century are to be adequately addressed.

The major rate-limiting step in sea-air gas exchange is slow molecular transport across the so-called “surface microlayer”, a region only tens of microns thick at the sea-air interface formed by chemical and microbiological components. This environment is microbiologically distinct from the underlying water, containing enhanced populations of marine bacteria that have only been identified in recent years following advances in molecular identification. In such a microbiologically rich environment, active bio-cycling of gases such as CO₂ and CH₄ might reasonably be expected to impact their sea-air transfer rates, but this has never previously been demonstrated.

We examined directly the microbiological nature of the sea-surface microlayer for the first time and evaluated its potential for modifying the sea-air flux of CH₄, using our expertise in molecular microbiology and air-sea gas exchange.

Microlayer (neuston) and subsurface (pelagic) seawater samples (North Sea) were collected and subsequent DNA extracts used to construct clone libraries and for subsequent identification of microbiologically active sites specific to CH₄-oxidising microbes. The results showed significant differences in the microbial communities

of the neuston and pelagic samples, with *Vibrio* and *Pseudoalteromonas* spp. dominating the neuston layer.

We subsequently examined the potential role methanotrophic bacteria in the sea-air exchange of CH₄ in controlled experiments in a laboratory gas exchange tank in which the CH₄ compositions of the air and water phases and the methanotroph content of the water could be selected and modified. Our results showed a small but significant enhancement of sea-air CH₄ exchange in the presence of methanotrophic bacteria. This suggests that a previously ignored, small bacterial consumption term should be taken account of in sea- air CH₄ exchange and that similar “sinks” may apply for other trace gases at the sea surface. If so, current gas exchange models may include errors that could potentially compromise global trace gas budgeting.

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Finally, the biggest thanks go to my family and friends who have contributed financial support and general distraction, I think it was worth it. Special mention to the three (don't forget to look behind you) barbarians and Leicester Hospitals Mixed Hockey club.

I'm off skiing now.

Declaration

The work presented in this thesis is original research conducted by myself, unless otherwise stated, under the supervision of Prof. JC Murrell at the University of Warwick and Dr. R Upstill-Goddard and Prof. NJP Owens at the University of Newcastle upon Tyne. All sources of information have been acknowledged by means of a reference. The experimental work described in this thesis, including work in conjunction with Tom Frost (sections of Chapter 4), is original work and has not been submitted for a previous degree at this or any other university. The research was funded by a NERC studentship.

Mark Franklin.

Chapter One

Introduction

1.1. Introduction

Most biological and chemical processes of importance occur at surfaces or interfaces between differing environments (Hardy, 1982). Covering around 70% of the world's surface, the air-sea interface controls the exchange of natural and man-made substances between the atmosphere and the hydrosphere.

Processes occurring in the sea surface microlayer make it more important than its thin slice of the water column might indicate (Figure 1.1). Indeed, it has been suggested that it was at this interface that organic molecules were first organised into the preliminary semblances of life over three billion years ago (Hardy, 1982).

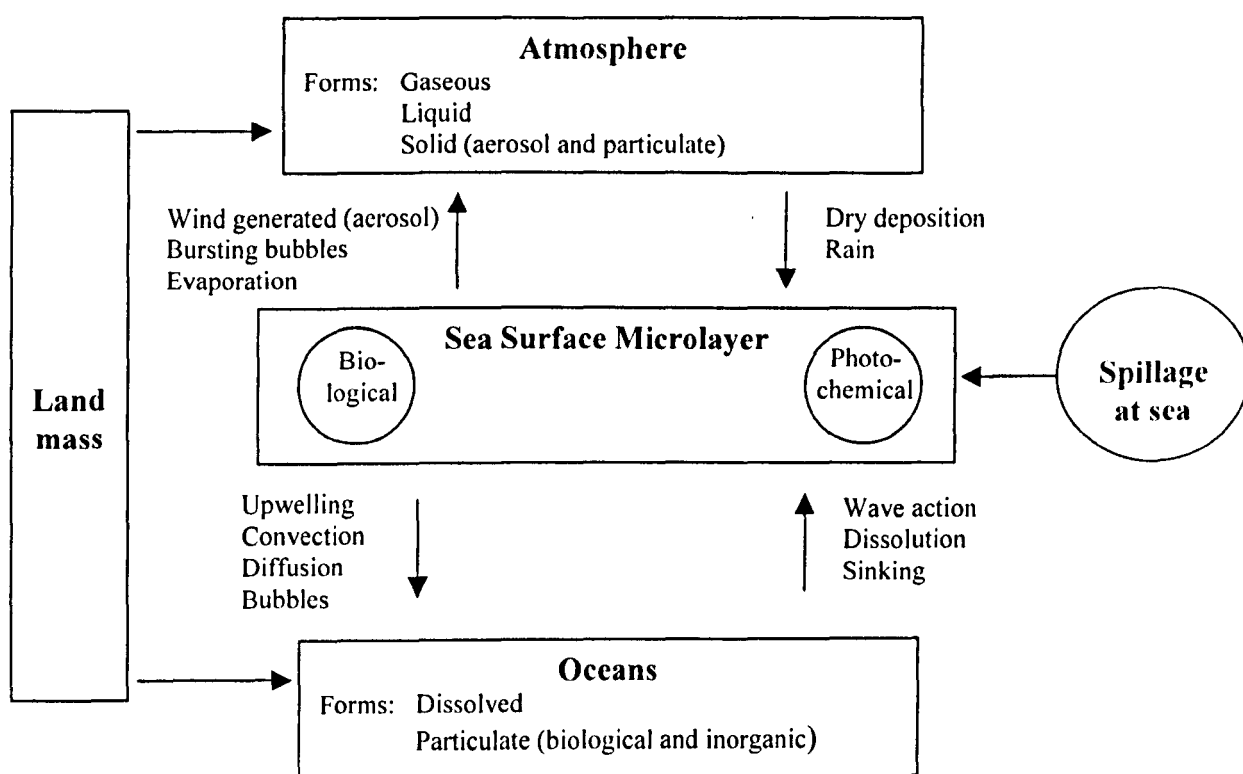


Figure 1.1. Sources and sinks of natural and man-made materials and the sea surface microlayer (from Liss, 1975).

The air-water interface and associated surface microlayers of marine and inland waters represent a unique habitat for microorganisms when compared to the respective bulk waters (Maki, 1993), biological activity at this interface is also greater than in bulk water (Hardy, 1982). Collectively organisms within the microlayer are known as the neuston (Naumann, 1917). The community of bacteria, which is present in the sea surface microlayer, is known as the bacterioneuston.

Compared to the water only a few centimetres below the sea surface, the microlayer blooms with bacteria (Sieburth, 1971; Sieburth *et al.*, 1976; Tsyban, 1971; Harvey, 1966; Morita and Burton, 1970; Bezdek and Calucci, 1972; Munster *et al.*, 1998). Bacteria in the bacterioneuston have commonly been found to be 10^2 - 10^4 fold more abundant than the bacterioplankton of the water column. For example, microlayer samples collected from the west coast of Sweden yielded enrichment factors (microlayer concentration/ 10cm below the surface concentration) of between 1 - 10^4 for bacteria and from 10^2 - 10^4 for yeasts and moulds, (Kjelleberg and Hakansson, 1977). In a Swedish fjord, microlayer enrichments of bacterioneuston were greater than 100 fold compared to pelagic waters at all stations investigated (Kjelleberg, Stenstrom and Odham, 1979). Williams *et al.*, (1986) found an enrichment factor for bacteria in the sea surface microlayer of 1.3-2.0 in the Southern Gulf Of California and the west coast of Baja, California.

Enriched levels of dissolved organic carbon have often been found in surface microlayers (Sieburth *et al.*, 1976; Hardy, 1982). During the cruises of Williams *et al.*, (1986) they found mean enrichment factors of: 1.1-2.4 for soluble inorganic nutrients, dissolved organic carbon, nitrogen, urea, carbohydrate and lipid; 1.3-2.0 for ATP, chlorophyll-a, microplankton and bacteria; and 1.1-3.7 for particulate carbon and nitrogen and both dissolved and particulate protein.

The organic compounds are believed to originate from exudate material released by plankton and are brought to the surface by rising bubbles, floatable particulates and migrating and excreting phagotrophic protists. Dissolved substances, particles and microorganisms are also brought to the interface by simple diffusion (Garrett, 1967), convection and upwelling from sediments and subsurface waters. At the same time, the microlayer is a sink for fall out from the atmosphere (Duce *et al.*, 1976) and so as Norkrans (1980) states, it is an accumulation layer.

1.2. What is the sea surface microlayer ?

Surface microlayer is a loosely defined term often implying no particular thickness, chemical composition, or concentration, but simply referring physically to the thin surface layer collected by the particular sampling device used.

The sea surface microlayer has been operationally defined to include the uppermost 1000 μm of the sea surface and the lower 50-500 μm of the atmosphere (Gesamp, 1995). What is already known and what will be learned in the future, regarding the microlayer and the bacterioneuston, has been, and will be somewhat influenced by the sampling procedure employed. The ultimate aim of sampling the air-water interface is to collect only the organisms at the interface and leave all subsurface water behind (Kjelleberg, 1985). In most, if not all, of the investigations on this environment, the surface film is operationally defined based upon the depth of the sample layer collected, which is, in turn, dependent upon the sampler employed (Maki, 1993). A number of different sampling techniques are presented in Table 1.1.

Table 1.1. Examples of surface film sampling devices, taken from Maki (1993).

Sampling method	Depth sampled
Paper/membrane filter	8-100 μm
Screen sampler	300-400 μm
Rotating drum	34-100 μm
Bubble microtome	0.5-10 μm
Germanium prisms	30 nm
Glass plate	20-100 μm
Teflon plate	50-100 μm
Freezing probe	1000 μm
Polyvinylchloride film	1 μm

In 1999, Falkowska tested three different sampling devices and measured the mean microlayer thickness picked up by each.

Sampling device:	Thickness of microlayer
Teflon Plate	10 μm
Glass plate	90 μm
Screen	250 μm

Sampling was carried out under variable weather conditions and the microlayer thickness was found to increase with wind speed, although when the wind speed exceeded 8 ms^{-1} the microlayer thickness was observed to decrease gradually. As can be seen when comparing the figures in Table 1.1 with those by Falkowska, the thickness of sample varies between samplers and individual sampler quite significantly. Thus, each sampler must be calibrated for individual experiments and the samplers must be characterised to a higher degree within procedures, as, for example, there is no uniform glass plate.

Several factors are important in the choice of sampler and consequently in the evaluation of surface microlayer data. These include the needs of the investigator, the volume of sample required to complete all the desired analyses and the type of material the sampler is constructed from. This last factor is particularly important for those sampling devices that function through adsorption of the surface film because different materials will adsorb different components (Garrett and Duce, 1980). Most sampling techniques have been developed by investigators looking at chemical composition rather than biological composition of the sea surface microlayer (Norkrans, 1980), and so this must be considered when selecting a sampler to investigate the biological composition of the environment.

The bacterioneuston layer has been suggested to be of the order of 1 μm thick (Sieburth *et al.*, 1976, Norkrans, 1980, Hermansson, 1990). However, Sieburth (1983) has indicated that microcolonies of bacteria may form layers that are several micrometers thick at the surface. The thickness of the bacterioneuston layer may also vary due to the surface-active properties of the bacteria involved (Maki, 1993). It is apparent that most of the samplers mentioned in Table 1.1 will collect a layer of surface water containing the bacterioneuston.

The presence of organic films, as well as the surface tension forces of the interface itself, provide an area of physical stability where compounds, particulate materials and organisms can concentrate (Hardy, 1982). Figure 1.2 describes a model of the sea surface microlayer.

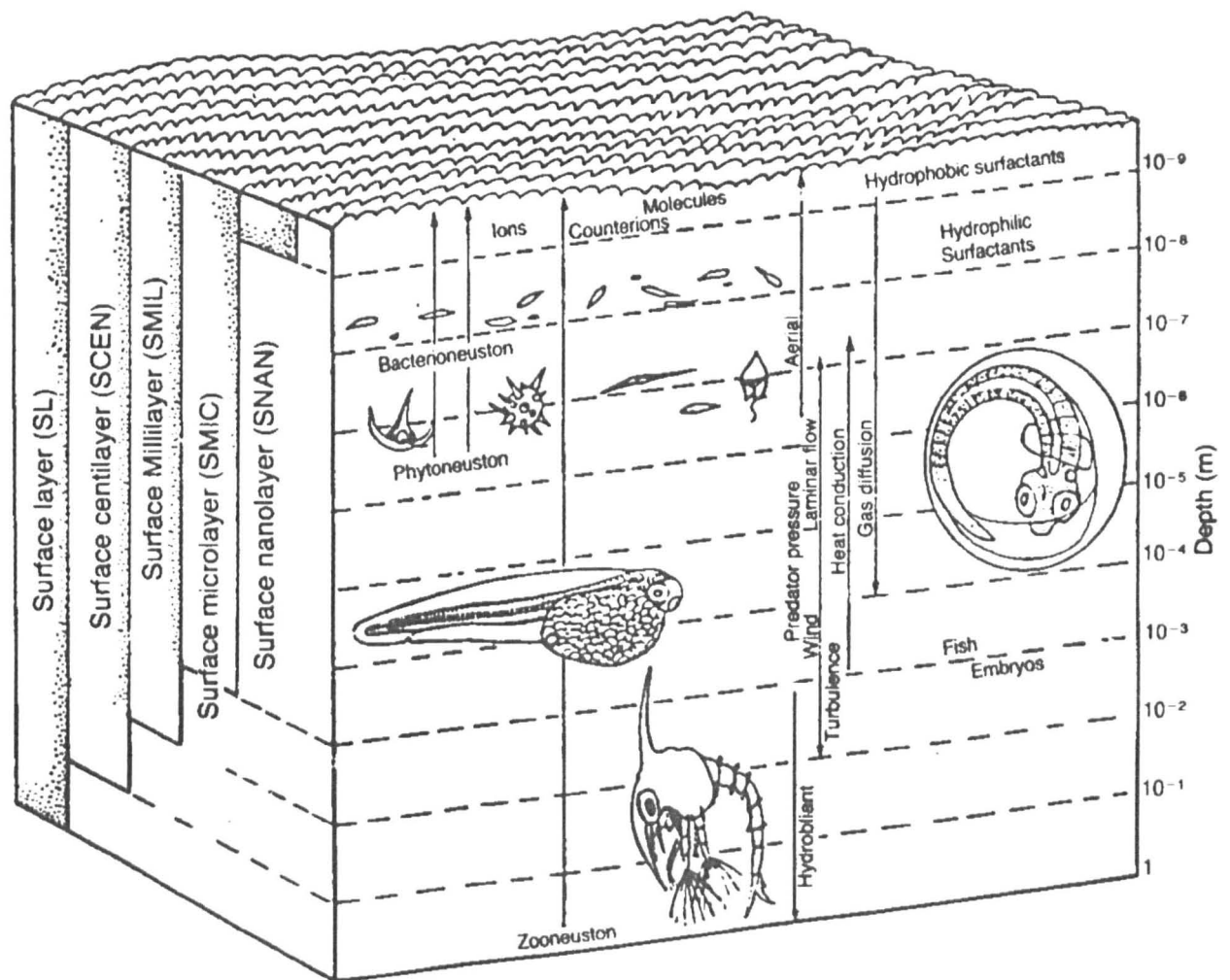


Figure 1.2. Conceptual model of the sea surface layer. (Taken from Liss & Duce, 1997).

A general hypothetical model considers that the aquatic surface layer (SL or upper 1 metre of the water surface) actually consists of a series of sublayers or microstrata of different thickness, each with different physical, chemical and biological characteristics. These microstrata include the surface nanolayer ($<10^{-6}$ m) highly enriched in organic compounds; the sea-surface microlayer ($<10^{-3}$ m) with high densities of microorganisms (bacterioneuston and phytoneuston); the surface millilayer ($<10^{-2}$ m) primarily small crustaceans and the eggs and larvae of fish and

shellfish (zooneuston); and the surface centilayer (10^{-1} m) with larger crustaceans and floating organisms.

1.3. Microlayer stability

In comparison to the water column, the sea surface microlayer can be characterised as a more physically stable but climatically variable environment. Physical stability results from strong surface tension forces. Normal oceanic waves and ripples cause periodic changes in the thickness of the microlayer, but generally leave the microlayer intact. Breaking waves will disrupt the microlayer temporarily, but white caps cover only 3-4% of the ocean surface at any one time (MacIntyre, 1974). Langmuir circulation tends to concentrate organic materials to form surface films, and if disturbed or mixed, these films rapidly re-establish their integrity. Formation and reorientation of organic surface films at the air/water interface has been estimated to occur in only approximately 0.2 seconds (Dragcevic and Pravdic, 1981). Langmuir circulation, first described by Irving Langmuir in 1938, is a coherent wind-driven motion in the upper layers of oceans and lakes, consisting of quasi-steady parallel horizontal vortices with axis generally aligned to the wind direction. The disparities in microlayer chemical and biological composition found by different workers probably result from three primary causes. First, the patterns of biological and chemical enrichment may be highly clumped in their distribution and problems of sampling scale have not been adequately addressed. Second, the numerous sampling techniques each collect a different type of sample. Third, improved methods of detection allow greater investigation.

In Hardy's estimation (1982) much of the data at that time could be resolved by a model in which the upper layer consists of a monomolecular lipid film of 10-20

Å in thickness, below which is a polysaccharide protein layer ranging in thickness from 100–300 Å and a closely associated layer of abiotic particulate matter, bacterioneuston and yeasts and moulds to a depth of 5 µm. In all probability, naturally occurring sea surface films or microlayers vary in thickness anywhere from tens of Angstroms to tens of micrometers, depending upon microlayer formation, turbulence at the interface and the chemical nature of the surface-active organic compounds.

1.4. The sea surface microlayer as an extreme environment

Although there is no absolute definition of the term “extreme” environment, they have usually been described in terms of environmental parameters or the organisms found there. In the first case, extreme has been suggested to be the ends of environmental gradients. For example, temperature, salinity, pH, nutrients, and pressure (Vestal and Hobbie, 1988). In the second case, extreme has been suggested to be associated with a decrease in diversity of organisms (Edwards, 1990).

With the sea surface being subject to environmental and climatic variations, salinity changes, temperature fluctuations, increased levels of solar radiation and accumulation of toxic substances (for example, heavy metals, pesticides), but not necessarily with one factor at the limit of an environmental gradient, it has been labelled a “fluctuating” environment (Shilo, 1979). Maki (1993) considered the air-water interface to be an extreme environment and argued that because of its position, at the very top of the water column, it bears the brunt of these fluctuations and is affected when the rest of the water column may not be.

A number of investigators have reported petroleum hydrocarbons, polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and

pesticides in the surface film. In addition, dissolved phenolic compounds have also been demonstrated to be enriched in surface films (Hardy, 1982; Norkrans, 1980; Maki, 1993).

The advantages for the bacterioneuston of being associated with the air water interface, particularly in oligotrophic bulk waters, is the presence of nutrients. Enrichments of either dissolved organic carbon (including proteins, polysaccharides, and lipids), phosphate, ammonia, nitrate, or nitrite have been reported from surface films and foams (Maki, 1993). Sieburth *et al.* (1976) suggested that nutrient concentrations in samples collected from surface films and adjusted to a microlayer thickness of 0.1 μm approach those concentrations of nutrients found in laboratory cultures.

1.5. Bacterioneuston

Although there are a number of reports regarding the numbers and activity of the neuston, there is not much known about what constitutes a “successful” bacterioneuston. Discussion, therefore, of the bacterioneuston is limited in the sense that many characteristics that may be important to their survival have not necessarily been demonstrated or even looked for in bacterioneuston isolates.

The air-water interface with its adsorbed macromolecules has been suggested to “appear” to a bacterium as a solid substratum (Sieburth, 1983; Kjelleberg, 1985). As such, it is not surprising that cell surface characteristics like hydrophobicity and charge, that are important in adhesion of bacteria to solid substrata appear to play a similar role in bacterial adhesion to the air-water interface (Kjelleberg and Stenstrom, 1980; Dahlback *et al.*, 1981; Hermansson *et al.*, 1982, Kjelleberg, 1985). Furthermore, the presence of extracellular polymers, fibres, and fimbriae observed to

be associated with bacterioneuston (Fuerst *et al.*, 1987) may mediate firm adhesion of the bacteria to the air-water interface as they do to solid substrata (Marshall, 1985).

In 1993, Maki reviewed a number of articles concerning the bacterioneuston and summarised what was known about bacteria within the neuston:

Adenosine triphosphate (ATP) enrichment indicates that there is a living microbial biomass in surface films.

Measurements of colony forming units and direct counts of respiring bacteria indicate that a portion of the bacteria are viable.

These bacteria apparently possess the enzymatic capability (for example, proteolytic and lipolytic) to utilise surface film organic material.

These points suggest that a portion of the bacterioneuston is capable of active metabolism in spite of any inhibitory environmental stress.

Conrad and Seiler (1988) suggested that the bacterioneuston exhibit an affect upon gaseous exchange and transport mechanisms from the water column to the atmosphere and vice-versa.

1.6. Numbers

The potential range and diversity of microbial populations within the bacterioneuston is immense. There has been controversy as to whether bacterial numbers and, more importantly, bacterial activities might be higher in the surface microlayer compared to bulk seawater (Norkrans, 1980; Conrad and Seiler, 1988).

Studies of the bacterioneuston have shown, enrichment of microorganisms and activities in the surface microlayer (Hardy and Apts, 1984), an enrichment of respiratory electron transport activity (De Souza, 1985), and an increase in the

degradation capacity for low substrate concentrations (Hermansson and Dahlback, 1983). Kjelleberg and Hakansson, (1977) stated that bacterial numbers are considerably greater in the surface microlayer than in subsurface water with estimates ranging up to a 10,000 fold increase. Munster and coworkers (1998) found the biomass of the bacterioneuston to be enriched 1.5 to 2-fold when compared to the bacterioplankton. Consequently it appears reasonable to assume processes to be active in the sea surface microlayer which affect the net exchange of gases between ocean and atmosphere.

1.7. Bacterial composition of the bacterioneuston

From a taxonomical point of view, the bacterioneuston is very poorly characterised. When the bacteria have been identified, the genus *Pseudomonas* has repeatedly been reported as dominant; for example, by Sieburth (1971) in the Caribbean Sea and in the Atlantic and Pacific Oceans, and Tsiban (1975) in the Black Sea, the Sea of Azov, the Caspian Sea and Alaska Bay. The latter author also found the genera *Chromobacterium* and *Micrococcus* generally distributed. Carty and Colwell (1975) classified bacterial isolates from the air-sea interface during a Pacific Ocean expedition. Besides *Pseudomonas* species, they found *Vibrio*, *Aeromonas*, and *Spirillum* species to be predominant. The same species were also found in the air above the sea surface, transferred to the air by bursting bubbles and foam.

1.8. Gas exchange across the air-water interface

Gas exchange between the atmosphere and the hydrosphere is one of the fundamentally important processes in biogeochemistry. Surface films may block gas exchange across the air-water interface in two ways: directly, in which the film is an additional static barrier to the passage of gas molecules, and indirectly, in which the film alters the interface, and as a result, factors that affect gas exchange are altered in magnitude (Liss, 1977).

Probably because of its small size ($\sim 10^{-4}$ % of the total euphotic zone volume,) the microlayer has previously received very little attention as a potential site for modifying material transfer between water and atmosphere.

Oceans are a net atmospheric source for many gases of biogeochemical interest. Examples include gases such as dimethyl sulfide (Turner *et al.*, 1996), nitrous oxide (Law and Owens, 1990), methane (Owens *et al.*, 1991) and carbon monoxide (Johnson and Bates, 1996). Such gases have an important role in atmospheric chemistry and climate regulation and in each case, the gas exchange rate itself directly controls the sea to air flux. Therefore, knowledge of gas exchange rates across the air sea interface is directly relevant to a wide range of global issues.

In a basic conceptual model of air-water exchange, the interface between the two environments can be visualised as a simple layered system with gas transport effected by both molecular diffusion and turbulent mixing. In this model, beyond several tens of μm from the interface on either side, gas transport is dominated by rapid eddy motions as the rate of turbulent transport is several orders of magnitude greater than the rate of diffusion (Frost and Upstill-Goddard, 1999). However, closer to the interface, turbulent transport becomes progressively attenuated because of the viscous properties of the boundary layer. So that molecular diffusion takes over as

the dominant transport mechanism the closer to the interface we get, giving rise to a 'stagnant' boundary layer (sometimes referred to as the "stagnant film") on each side of the interface (Conrad and Seiler, 1988). Thus, for unreactive trace gases with measurable aquatic sources or sinks, a gas concentration gradient is developed across each of the two stagnant boundaries. This model assumes direct proportionality of the transfer velocity of a gas to its diffusivity. All models of gas transfer velocity assume that the transfer velocities of the different gas species are only dependent on their different diffusion properties. They do not allow for other effects such as chemical or biological reactions at the water surface that might alter the transfer velocities of biologically and/or chemically active gases to different extents.

Sulfur hexafluoride, for example, is chemically and biologically unreactive so that its flux across the air water interface most probably is exclusive to its gradient. This is not necessarily the case for other, non-conservative gases like carbon monoxide, hydrogen, methane, and nitrous oxide, which are highly reactive and biologically transformable (Conrad and Seiler, 1988).

The microbial interaction at gas-liquid interfaces in microbial ecology has been studied much less than at solid-liquid interfaces in aquatic systems. At both interfaces, the microbial interaction is dependent on several similar forces. A solid substrate, however, partly fixes the solid-liquid system, whereas an air-water interface consists of an organic layer on a dynamic water surface whose chemical composition is constantly being modified by physical, chemical, and biological processes. For this reason, simple generalisations to predict the interaction at natural air-water interfaces are very difficult (Norkrans, 1980).

Previous work has uncovered circumstantial evidence for the microlayer as a site of biogeochemical gas flux modification, most notably for carbon dioxide, carbon monoxide and methane (Conrad and Seiler, 1988).

1.8.1. Schmidt number

When investigating different gases and then comparing between each, certain variables need to be taken in to consideration, one way to do this is using certain factors such as Schmidt numbers. This factor incorporates the effects of temperature and salinity into an equation (Wannikhof, 1992). The relationship between the k_w (gas transfer velocity) values for any two gases may then be stated in terms of their Schmidt numbers (Sc):

$$\frac{k_{w1}}{k_{w2}} = \frac{(Sc_2)^n}{(Sc_1)^n}$$

where n , the so called “Schmidt number exponent”, is a function of the film thickness (Frost, 2000).

1.9. Why study methane?

The exchange of gases between these two differing environments, the hydrosphere and the atmosphere, has an important effect on climatically important gases such as carbon dioxide and methane. Supersaturation of methane in surface water (the upper 300 m of the water column) is a persistent feature of most ocean waters (Lamontagen *et al.*, 1973, Cicerone and Oremland, 1988). The global oceanic source of methane to the environment has been estimated at 5-50 Tg yr⁻¹, which is 3-

30% of all natural sources of methane (Holmes *et al.*, 2000). The large uncertainty in the estimates of the magnitude of the marine source of this gas, which is 3.7 times more effective per mole at trapping radiated heat than CO₂ (Lashof and Ahuja, 1990), emphasises the need for better understanding of the variations of the methane cycle.

In addition to methane, it has been suggested that at least 15 one-carbon (C₁) compounds can be found in the marine environment making it a potential habitat for many methylotrophic organisms (Kiene, 1993).

1.10. Model systems

Model systems lack the multiplicity of a natural ecosystem, but they offer great advantages. Environmental factors may be kept constant, and individual variables can be studied under controlled conditions. In the case of interface studies, they seem especially justified since the initial events of interaction are governed by purely physiochemical forces, in which the microorganisms behave as colloid particles, their biological activity being manifested only in subsequent stages (Norkrans, 1980).

1.11. Overview of gases used in this study

1.11.1. Sulfur hexafluoride

SF₆ is a fully fluorinated compound with a very high Global Warming Potential (GWP) (23900, where CO₂=1). Almost all SF₆ in the environment is man-made (by passing free fluorine gas over molten sulfur). SF₆ is very insoluble and although at high temperatures (>204 °C) it decomposes to form toxic fluoride

compounds, it is inert under normal environmental conditions, which is reflected in its extremely long atmospheric lifetime, 3200 years (Houghton *et al.* 1996).

The atmospheric background concentration of SF₆ in 1980 was ~0.6 pptv (Lovelock and Ferber, 1982), but had risen to ~1.6 pptv by 1985 (Watson and Liddicoat, 1985) and was recently reported to be as high as ~3.6 pptv in 1998 (Frost and Upstill-Goddard, 1999).

1.11.2. Nitrous oxide

The atmospheric lifetime of Nitrous Oxide (N₂O) is approximately 120 years, with its GWP around 300 times that of CO₂. N₂O is responsible for ~6% of anthropogenic greenhouse warming (Schimel *et al.*, 1996). Atmospheric N₂O concentrations have increased from ~275 ppbv during the pre-industrial era to ~312 ppbv in 1995 (Schimel, 1998).

N₂O is mainly produced from the microbially mediated processes nitrification and denitrification. During nitrification, reduced nitrogen (typically ammonia) is converted to an oxidised form, mainly nitrate, by heterotrophic oxidising bacteria. However, this process is not 100% efficient. An estimated 0.3% of the nitrogen is not fully oxidised, but is converted to N₂O (Capone, 1991). Denitrification describes the reduction of oxidised inorganic nitrogen to dinitrogen gas in the absence of oxygen. Where nitrate or nitrite is used as a terminal electron acceptor. This respiratory process yields ~1-6% N₂O (Nevison and Holland, 1997).

Although N₂O has a long atmospheric lifetime due to its slow sink (photolysis and oxidation in the stratosphere) it has important implications in atmospheric chemistry, being the greatest producer of stratospheric NO_x (Vitt and Jackman, 1996) and the primary source of anthropogenic stratospheric ozone

depletion (Houghton *et al.*, 1996). In the troposphere, N₂O is relatively inert and is involved in the formation of low level ozone, both a toxin and greenhouse gas (Lee *et al.*, 1995).

1.11.3. N₂O in the oceans

The contribution of the worlds oceans to the global emissions of N₂O was estimated at ~13% (Khalil and Rasmussen, 1992). Although most of the ocean appears to be near equilibrium (the mean global ocean saturation is 103%, Bange *et al.*, 1996), it is far from a uniform ecosystem and an estimate of the oceanic emissions has to take into account the variability of this environment. Enhanced N₂O concentrations have been found in the surface waters of biologically active regions, especially those with a pronounced low-oxygen subsurface layer, for example, the Arabian Sea (Law and Owens, 1990).

1.11.4. Methane

Methane is the simplest compound in the alkane series and the main constituent of natural gas. It is the most abundant organic gas and hydrocarbon in the atmosphere. Methane is a relatively insoluble, but chemically and radiatively active trace gas (Cicerone and Oremland, 1988; Crutzen, 1991) and is of great importance as it is a potent greenhouse gas.

Carbon dioxide (CO₂) is generally recognised as the biggest cause of global warming, but in this respect, a CH₄ molecule is around 30 x more effective as a warming agent than a CO₂ molecule (Dickinson and Cicerone, 1996; Ramanathan *et al.*, 1985).

It has been estimated that in the past century, CH₄ has accounted for about 15-25% of the thermal trapping while CO₂ has contributed approximately 60% (Ramanathan *et al.*, 1985; Rodhe, 1990).

A rise of ~245% in atmospheric CH₄ concentration has been observed from the pre-industrial value of ~700 ppbv to ~1720 ppbv in 1994 (Houghton *et al.*, 1996). Rates of atmospheric CH₄ increase have fluctuated significantly over the last few decades, from a high of ~20 ppbv per year in the 1970's to a zero growth rate in 1992 and a rate of ~8 ppbv per year in 1993 (Dlugokencky *et al.*, 1994). The brief period of quiescence in the CH₄ growth rate during 1992 has been ascribed to the collapse of the USSR and associated downscaling of industry.

Methane has an atmospheric lifetime of ~12 years, however, this time is dependent upon feedback mechanisms. The major sink for methane is reaction with stratospheric hydroxyl radicals (OH), which are subsequently reduced with increasing methane emissions. As a result, each mole of CH₄ emitted increases the atmospheric residence time of that mole by 50% (Lashof and Ahjua, 1990). Complicating the feedback mechanism however, is the event following stratospheric oxidation of methane, carbon dioxide and water vapour are produced (Crutzen, 1983, 1995), which are also greenhouse gases. Carbon isotope measurements suggest that ~20% of the total CH₄ emissions are from the thermogenic sources due to the production and use of fossil fuels (Houghton *et al.*, 1996), however, the contribution of natural sources (seep sites) to this may have been underestimated. The total thermogenic CH₄ source strength is thought to be ~4 times lower than the biogenic source (Houghton *et al.*, 1996). The mismatch between methane sources and sinks demonstrates the level of uncertainty involved in such measurement. Notwithstanding these uncertainties, a reduction of ~8% of current anthropogenic

emissions is believed to be required to stabilise atmospheric concentrations at the present level.

In aerobic environments methane is oxidised to carbon dioxide by methanotrophic bacteria. The process of biogenic methane production is, however, strictly anaerobic and microbially mediated. The producers of biogenic methane, the methanogenic bacteria, utilise acetate, carbon dioxide and hydrogen as substrates for methane production.

Wetlands are the greatest natural source of biogenic methane due to the high level of organic matter and anaerobic conditions. Natural wetlands are believed to contribute ~20% of methane to global emissions (Houghton *et al.*, 1996). Vast areas of wet rice paddies act as anthropogenic 'wetlands' also producing significant levels of biogenic methane. However, suitable conditions for biogenic methane production can be found in many different places: in wet soils, lakes, peat areas, the continental shelf, the digestive tracts of many animals and waste sites. Biogenic methane production is subject to a theoretical positive feedback mechanism associated with temperature. As global temperatures rise due to increasing methane, this may feed back to stimulate increased methane production from the temperature-dependent methanogenic bacteria (Houghton *et al.*, 1996).

An amount equal to 90% of the annual emissions is oxidised through photochemical reactions mediated by hydroxyl radicals in the troposphere. It is estimated that concentrations of methane in the atmosphere will reach 2.1-4.0 ppm by the year 2050 (Ramanathan *et al.*, 1985).

Recently, however, there has been a reported decrease in methane emissions (Steele *et al.*, 1992; Dlugokencky *et al.*, 1994). This is possibly due to a number of factors including increased atmospheric concentrations of OH radicals (Prinn, 1994).

changes in fossil fuel sources and slowing of agricultural practices involving rice farming and cattle farming (Khalil and Rasmussen, 1993).

1.12. The oceanic methane paradox

The methane found in anoxic environments can arise from either the bacterial decomposition of organic matter in the water column (or sediment), or from seepage from surrounding rock that may contain methane (Atkinson and Richards, 1967).

Methane can be formed by bacteria either from carbon dioxide reduction or acetate fermentation (McCarty, 1964, Pine and Barker, 1956) under anoxic conditions.



Although the process of CH₄ production is microbially mediated and strictly anaerobic, the ocean surface is highly oxygenated and is also typically supersaturated with CH₄, when compared to the atmosphere (Scranton and Brewer, 1977). This phenomenon appears to occur in a wide variety of geographical locations, making it a world wide phenomenon (Conrad and Seiler, 1988). This has been termed the “oceanic methane paradox”. Methylotrophs could therefore make a significant contribution to oceanic carbon cycling.

Table 1.2. Global methane budget for 1995 (Houghton *et al.*, 1996).

Sources		Tg CH ₄ yr ⁻¹	Range (Tg CH ₄ yr ⁻¹)
Natural	Wetlands	115	55-150
	Termites	20	10-50
	Oceans	10	5-50
	Other	15	10-40
Anthropogenic	Fossil Fuel related	100	70-120
	Cattle (enteric fermentation)	85	65-100
	Rice Paddies	60	20-100
	Biomass Burning	40	20-80
	Landfills	40	20-70
	Animal Waste	25	20-30
	Domestic Sewage	25	15-80
	Total Sources	535	210-580
Sinks			
	Hydroxyl radicals	490	405-575
	Stratospheric removal	40	32-48
	Atmospheric increase	37	35-40
	Removal by soils	30	15-45
	Total sinks	597	127-708

Some possible pathways for CH₄ production in oxygenated waters are anaerobic microenvironments such as faecal pellets and zooplankton (Marty, 1993), with sinking particles acting as transport mechanisms (Karl and Tidbrook, 1994). CH₄ production has therefore been linked to high phytoplankton biomass (Owens *et al.*, 1991). Even though the methane source of the oceans is believed to be small relative to the other sources, there is spatial variability. Areas such as the Arabian Sea have been identified as having high methane concentrations associated with upwelling which, given the right conditions, were calculated as accounting for 133% of the current estimates of the open-ocean source of methane (Owens *et al.*, 1991).

1.13. Methane oxidising bacteria

Methane oxidising bacteria are known as methanotrophs and are a subset of the physiological group of bacteria known as the methylotrophs. Methylotrophs can be defined as microorganisms that are capable of growth on one-carbon compounds containing one or more carbon atoms but no carbon-carbon bonds. Methanotrophs are a group of Gram-negative bacteria that utilise methane as a sole source of carbon and energy. The biochemistry of methylotrophs has been described in detail (Anthony, 1986).

The first methanotroph, *Bacillus methanicus*, was isolated in 1906 (Söhngen, 1906). Though the basic scheme of classification was established after Whittenbury and co-workers (Whittenbury *et al.*, 1970) isolated and characterised over 100 new methane oxidising bacteria. Presently there are 10 recognised genera of methanotrophs namely, *Methylomonas*, *Methylobacter*, *Methylomicrobium*, *Methylococcus*, *Methylosinus*, *Methylocystis*, *Methylocaldum*, *Methylosphaera* (Bowman *et al.*, 1993), *Methylocella* and *Methylosarcina* (Wise *et al.*, 2001). These 10 genera can then be further split into two broad groups; Type I and Type II based on physiological and phylogenetical properties. A summary of these properties can be seen in Table 1.3 for the 8 most well characterised genera.

Type I methanotrophs cluster in the gamma subdivision of the Proteobacteria and they utilise the ribulose monophosphate (RuMP) pathway for assimilation of formaldehyde into cell carbon (Quayle, 1972), possess bundles of intracytoplasmic membranes and have DNA with a mol % G+C of 43-60. Type I methanotrophs appear to lack a complete TCA cycle, indeed the absence of several enzymes of central metabolic pathways has been suggested as a reason for their inability to grow on multi-carbon compounds (Murrell and Holmes, 1995).

Type II methanotrophs cluster in the alpha subdivision of the Proteobacteria. They utilise the serine pathway for formaldehyde assimilation (Quayle, 1972), possess intracytoplasmic membranes arranged around the periphery of the cell and have a mol % G-C of 62-67.

There is also a Type X, which accommodates methanotrophs similar to *Methylococcus capsulatus* (Bath), which share some characteristics of both Type I, and Type II. The intracytoplasmic membranes mentioned here are very distinctive and similar formations are only seen in one other group of bacteria, the ammonia oxidisers (Hanson and Hanson, 1996).

Table 1.3. Methanotroph diversity, compiled from Bowman *et al.*, (1993, 1995)

	<i>Methylomonas</i>	<i>Methylobacter</i>	<i>Methylomicrobium</i>	<i>Methylococcus</i>	<i>Methylocaldum</i>	<i>Methylosphaera</i>	<i>Methylosinus</i>	<i>Methylocystis</i>
Number of validly described species	3	3	3	2	3	1	2	2
Phylogenetic group	gamma	gamma	gamma	gamma	gamma	gamma	alpha	alpha
Membrane Type	Type I	Type I	Type I	Type I	Type X	Type I	Type II	Type II
Major fatty acid type ^a	16:1	16:1	16:1	16:1	16:1	16:1	18:1	18:1
Pathway for formaldehyde assimilation	RuMP RuMP	RuMP	RuMP	RuMP/ Serine ^b	RuMP/ Serine	RuMP	Serine	Serine
Enzyme type	pMMO/sMMO	pMMO	pMMO	pMMO/sMMO	pMMO	pMMO	pMMO/sMMO	pMMO/sMMO
Mol% G+C content of DNA	51-59	49-54	49-60	59-66	57-59	43-46	63-67	62-67
N ₂ fixation	V	-	-	+	-	+	+	+
Pigmentation	Carotenoids	Brown/yellow	-	Brown/yellow	Brown	-	-	-

a *Methylobacter* and *Methylococcus* lack 16:1 ω8c (Bowman *et al.*, 1995).

b *Methylococcus* sp. Does not have all serine pathway enzymes.

1.13.1. Methane oxidation pathway

Methanotrophs oxidise methane, via methanol, to formaldehyde, which is assimilated into cell biomass or can be further oxidised to formate and subsequently to CO_2 . The pathway of methane oxidation is shown in Figure 1.3.

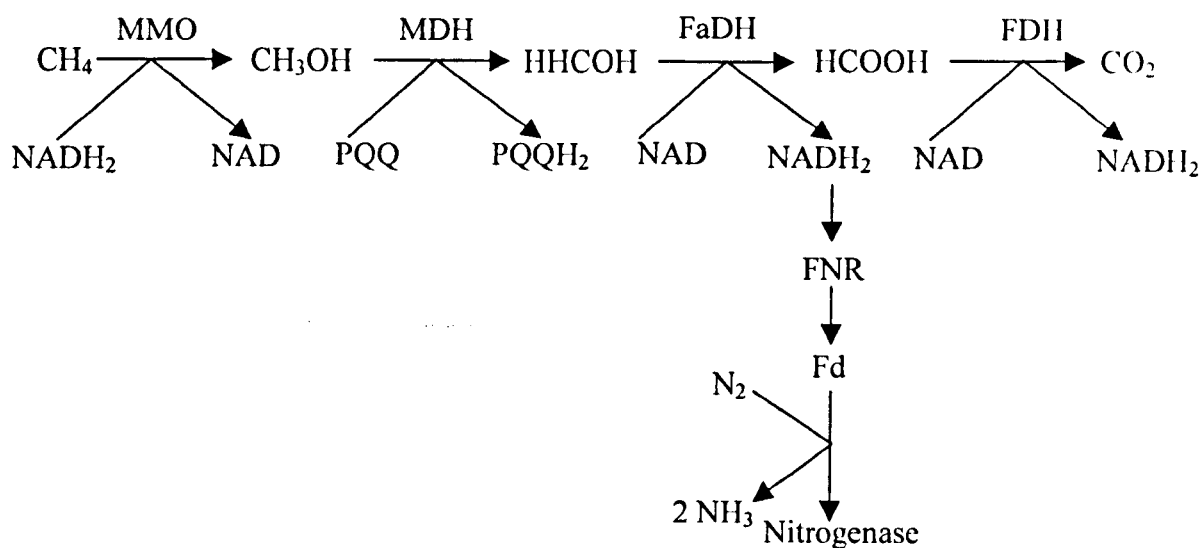


Figure 1.3. Oxidation of CH_4 coupled to the reduction of N_2 where PQQ/PQQH_2 are the oxidised forms, respectively, of pyrroloquinoline and quinone; **MMO**: methane monooxygenase; **MDH**: methanol dehydrogenase; **FaDH**: formaldehyde dehydrogenase; **FDH**: formate dehydrogenase; **FNR**: Ferredoxin- NAD^+ oxoreductase; **Fd**: Ferredoxin.

The initial step, where methane is oxidised to methanol, is catalysed by the enzyme methane monooxygenase (**MMO**). The enzyme responsible for the oxidation of methanol to formaldehyde is methanol dehydrogenase (**MDH**). The oxidation of formaldehyde to formate is coupled to the reduction of NAD^+ to $\text{NADH} + \text{H}^+$ which provides a source of reducing power for **MMO** and biosynthetic reactions or for example in the case of *M. trichosporium* OB3b, the NADH can be used as a source of electrons for nitrogenase.

1.13.2. Methane monooxygenase

The methane monooxygenase enzyme catalyses the first step of the methane oxidation pathway. This enzyme is unique to methanotrophs and is found in two different forms. The membrane-bound particulate form (pMMO) is present in all known methanotrophs (Dalton *et al.*, 1990). The soluble cytoplasmic form (sMMO) is only found in certain methanotrophs (Stainthorpe *et al.*, 1990) and only expressed under certain growth conditions. Although some methanotrophs are able to express both forms of the enzyme e.g. *Methylococcus capsulatus* and *Methylosinus trichosporium*, they are not expressed concurrently and are the products of separate genes. The expression of pMMO or sMMO is dependent on the copper: biomass ratio. At high copper: biomass ratios the pMMO will be expressed. Cultures expressing pMMO have higher growth yields on methane than cultures expressing sMMO. This is because the sMMO catalysed reaction requires the low potential electron donor ($\text{NADH} + \text{H}^+$) and the supply of this reductant is often growth limiting (Leak *et al.*, 1985; Leak and Dalton, 1986). At low concentrations of copper ions, the sMMO form is expressed (Stanley *et al.*, 1983; Prior and Dalton, 1985; Nielsen *et al.*, 1997; Kaluzhanaya *et al.*, 2001). It has been postulated that the synthesis of sMMO by some methanotrophs may be a survival mechanism in environments where copper levels limit the growth of methanotrophs, however this has yet to be proven (Nguyen *et al.*, 1994; Zahn and DiSpirito, 1996).

In the past, the sMMO enzyme has been well characterised and studied (Stainthorpe *et al.*, 1990; Lipscomb, 1994; McDonald *et al.*, 1997) whereas difficulties in purifying the pMMO enzyme complex have hindered its characterisation until quite recently (Semrau *et al.*, 1995; Zahn and DiSpirito, 1996; Stolyar *et al.*, 1999).

1.13.3. The particulate methane monooxygenase

The membrane-associated or particulate methane monooxygenase (pMMO) is expressed at high copper to biomass ratios. It is only recently that Zahn and DiSpirito (1996) successfully purified an active form of the pMMO from *Methylococcus capsulatus* (Bath). The enzyme consists of three major polypeptides with molecular masses of 47, 27 and 26 kDa. The 47 kDa protein is encoded by the *pmoB* gene, the 27 kDa protein by the *pmoA* gene and the 25 kDa by the *pmoC* gene. These genes are arranged in a cluster in the order *pmoCAB*. Hybridisation studies have suggested that methanotrophs contain multiple copies of genes encoding the 45 and 27 kDa subunits (*pmoB* and *pmoA* respectively) (Semrau *et al.*, 1995). Stolyar *et al.* (1999) has reported a complete sequence for two copies of the whole *pmoCAB* gene cluster and provides evidence of a third copy of the *pmoC*. The genetic organisation and numbers of gene copies for the *pmo* system are highly similar to those observed for *amo* genes in *Nitrosomonas europaea* (Norton *et al.*, 1996, Sayavedra-Soto *et al.*, 1998). The role of these multiple gene copies is not well understood in either methanotrophs or nitrifiers. Since the two clusters of *pmo* genes are so similar in sequence, it seems likely that the enzymes produced from each copy have a similar function. Therefore the role of the duplicate gene clusters may simply be to provide increased gene expression (Stolyar *et al.*, 1999). It may be that the two copies are important for different types of growth conditions, for instance under stress conditions that might be encountered in the natural environment. The role of the third copy of the *pmoC* gene is unknown, but it probably fulfills a function necessary for generating active pMMO from both *pmoCAB* clusters (Stolyar *et al.*, 1999).

Zahn and DiSpirito have developed a working model for the pMMO based on their findings. Evidence indicates that the catalytic site involves both iron and copper and that the majority of the copper associated with the pMMO appears to be loosely associated with the enzyme and may provide a secondary function such as stabilising the enzyme. The 27 kDa polypeptide is thought to contain the active site and can be labelled by ^{14}C acetylene, a potent inhibitor of the enzyme (Prior and Dalton, 1985).

The pMMO and sMMO forms are quite different from each other in many respects. They differ in substrate specificity as the pMMO has a much narrower substrate range than the sMMO (Burrows *et al.*, 1984). The pMMO is also sensitive to inhibitors that do not affect sMMO such as thiol reagents, metal chelators and electron transport inhibitors (Scott *et al.*, 1981; Dalton *et al.*, 1984, Dalton, 1992)) whereas the sMMO is sensitive only to acetylenic compounds and the metal chelator 8-hydroxyquinoline (Stirling and Dalton, 1977,1979). Acetylene inhibits both forms of the enzyme and acts as a suicide substrate (Prior and Dalton, 1985).

1.13.4. Soluble methane monooxygenase

The soluble methane monooxygenase (sMMO) is present only in the cytoplasm of some methanotrophs under certain growth conditions. Extensive research has been performed on this form of the enzyme and it has been well characterised. The sMMO enzyme consists of 3 components, Protein A, Protein B and Protein C. Protein A is composed of 3 subunits α , β and γ of 60, 45 and 20 kDa respectively. It is the hydroxylase component and contains a hydroxy-bridge di iron cluster that is the site of the monooxygenation reaction (Ericson *et al.*, 1988; Fox *et al.*, 1988). Protein B is a 16 kDa protein that acts as a modulator of the methane

monooxygenase reaction by forming a specific complex with the hydroxylase component (Green and Dalton, 1989; Fox *et al.*, 1993; Lipscomb, 1994). Protein C is a 39 kDa iron sulfur flavoprotein responsible for shuttling electrons from NADH to protein A (Lund *et al.*, 1985; Lund and Dalton, 1985).

The genes of the subunits of sMMO have been cloned and sequenced from *Methylococcus capsulatus* Bath (Stainthorpe *et al.*, 1990), *Methylosinus trichosporium* OB3b (Cardy *et al.*, 1991a,b.) and *Methylocystis* sp. Strain M (McDonald *et al.*, 1997). The genes were found to be clustered on the chromosome in the order *mmoX* (encoding for the α subunit), *mmoY* (β subunit), *mmoD* (Protein B), *mmoZ* (γ subunit), *orfY* (protein of unknown function) and *mmoC* (Protein C).

In the methanotrophs studied to date, DNA derived polypeptide sequence analysis indicates that these genes are highly conserved in all methanotrophs possessing sMMO. This conservation has enabled gene probes to be designed and exploited for the detection of other methanotrophs containing the sMMO.

1.14. Detection of methanotrophs

There are several different genes which can be used as genetic markers for methanotrophs and which have been targeted for probe design. The *mxoF* gene encodes the large subunit of the methanol dehydrogenase, the enzyme responsible for converting methanol to formaldehyde, and it is present in all known Gram-negative methylotrophs and methanotrophs. It is highly conserved with greater than 75% identity observed at both the DNA and amino acid levels. The *mxoF* gene has been characterised from *Methylobacterium extorquens* AM1 (Anderson *et al.*, 1990) *Paracoccus denitrificans* (Harms *et al.*, 1987) and *Methylobacterium organophilum* XX (Machlin and Hanson, 1988). Oligonucleotide probes have been designed for

PCR amplification of this gene (McDonald *et al.*, 1995) and have been proven as a useful marker for detecting the presence of methylootrophs within various environments e.g. woodland soils, peat, arable soils and marine environments (Holmes *et al.*, 1996; McDonald *et al.*, 1995; McDonald and Murrell, 1997).

The sMMO genes have also been amplified by PCR using specific primers designed for this gene by McDonald *et al.* (1995). These primers were used to target a variety of environmental samples and successfully retrieved sequences identical or nearly identical to known sMMO sequences. The primers have also been used to obtain sequences that were similar to extant methanotrophs.

However, the sMMO is not universal to all methanotrophs (McDonald and Murrell, 1997) and so a better functional gene probe for these organisms would be one based on pMMO, present in all extant methanotrophs (Murrell, *et al.*, 1998). Work has been carried out to identify PCR primers that detect *pmoA*, encoding one of the genes diagnostic for methanotrophs, the particulate methane monooxygenase (Holmes *et al.*, 1995b; Costello and Lidstrom, 1999). These PCR primers have successfully retrieved sequences of the correct alignment from all known methanotrophs tested plus environmental samples. The PCR primer set constructed by Costello and Lidstrom (1999) produced no product with ammonia oxidiser *Nitrosomonas europaea* DNA (which contains a similar gene *amoA*, encoding the active site polypeptide of a related enzyme ammonia monooxygenase) and has been classed as *pmoA* specific (Costello and Lidstrom, 1999).

1.15. Ecology of methanotrophs

It is generally thought that methane oxidising bacteria (methanotrophs) are ubiquitous in nature (Whittenbury *et al.*, 1970, Whittenbury and Dalton, 1981). This

is perhaps not surprising since methane is abundant, with about 500 to 800 million tons being produced annually on a global basis (Cicerone and Oremland, 1988). Methane is also super saturated in seawater (Ward *et al.*, 1987) and therefore it is surprising that only a few reports on the isolation of marine methanotrophs exist.

It is now generally accepted that methanotrophs form the major biological sink for methane in the biosphere and methane oxidising bacteria have been isolated from a variety of soils, sediments, freshwater, and saline environments.

Aerobic methane oxidation activity is generally at its maximum close to the oxic and anoxic interface of freshwater environments (Rudd and Taylor, 1980). This is generally the zone where methane and oxygen concentrations are optimum for methanotrophs.

1.16. Isolation of methanotrophs

Traditionally the methods that have involved the identification and quantification of methanotrophs in the environment have included variations on the classical enrichment technique of Whittenbury and others. The short comings of culture based techniques for microbial ecology are well documented (Ward *et al.*, 1992; DeLong, 1997) and for methanotrophs include the problem of slow growth or scavenging of non-methanotrophs on even “purified agar” plates which may cause further errors with these techniques. Estimates of biomass and activities of methanotrophs can be made by measuring the evolution of radiolabelled CO₂ and incorporation of labelled CH₄ added to the samples, but this would not allow analysis of community structure. Phospholipid analysis is another way of assessing the types of methanotrophs in the natural environment but this technique is relatively expensive, requires sophisticated equipment, and the database for liposaccharide

hydroxy fatty acid and the phospholipid fatty acid profile database for methylotrophs is not comprehensive. The detection and identification of methanotrophs in environmental samples has also been attempted using antibodies, which potentially is an attractive idea. However, antisera (to whole cells) must be prepared against culturable methanotrophs, which must then react with all methanotrophs of interest and not detect non-methanotrophs.

Isolation and culturing of methanotrophs is typically carried out at CH₄ concentrations of 10-50% (vol/vol) which is much higher than *in situ* concentrations. Typical enrichment and isolation techniques could select against those organisms that are oxidising atmospheric concentrations (2 ppm) of CH₄ in the environment and instead, work in favour of a relatively inactive population, which are more highly adapted to the increased concentrations.

The view that a population of organisms as yet uncultured or characterised, which oxidise atmospheric concentrations of CH₄, are different from the extant methanotrophs has been generally accepted. However, recent work by Dunfield *et al.* (1999) proposed that organisms closely related to the known Type II methanotrophs carried out atmospheric methane oxidation. It has also been proposed that the kinetic properties of these organisms are determined by the CH₄ concentration under which they are grown, suggesting that the extant methanotrophs have a high affinity for CH₄ are responsible for CH₄ oxidation at atmospheric concentrations.

Molecular techniques have been employed to try and detect methanotrophs which utilise atmospheric concentrations of CH₄. Holmes *et al.* (1999) used PCR primers specific for *pmoA* and *amoA* sequences to analyse DNA from soils that showed atmospheric CH₄ uptake. Holmes and co-workers (1999) concluded that by

using radiotracers and functional gene probes, evidence exists to support the theory that there are phylogenetically distinct populations of methane oxidising bacteria in soils with a high affinity for CH₄ and which form a novel group within the α -Proteobacteria methanotrophs.

1.17. Methane oxidation by ammonia oxidisers

There is a large amount of sequence similarity and likely structural similarity between the pMMO and ammonia monooxygenase (AMO) enzymes. These enzymes also have the ability to oxidise both methane and ammonia. Methanotrophs oxidise methane to carbon dioxide via a series of intermediates, assimilate carbon at the level of formaldehyde but do not fix CO₂ (see Figure 1.3). Ammonia oxidisers oxidise ammonia to nitrite to obtain energy for the fixation of CO₂ using ribulose biphosphate carboxylase/oxygenase (RuBisCO). On a cell dry weight basis, the specific rate of methane oxidation by ammonia oxidisers is less than 5% of specific rates observed with resting cells of methanotrophs. In turn the rates of ammonia oxidation by methanotrophs are much lower than those of the nitrifiers (Bedard and Knowles, 1989). It should also be noted that oxidation of methane by nitrifiers reduces oxidation of ammonia and oxidation of ammonia by methanotrophs reduces oxidation of methane and therefore has a detrimental effect on growth rates (Whittenbury *et al.*, 1970). This however, is not a concern in this study as the detection of methanotrophs used a primer set specific only for methanotrophs (Costello and Lidstrom, 1999).

The ability of methanotrophs to oxidise ammonia and ammonia oxidisers to oxidise methane has led to the hypothesis that ammonia oxidisers may have a role in the oxidation of methane in some environments and *vice versa* (Dalton, 1977; Roy

and Knowles, 1994). There is no simple approach to distinguish between CH₄ oxidation activity of methanotrophs and nitrifiers in soils since these organisms have a similar *K_m* for methane (in the micromolar range) and are inhibited by the same compounds e.g. acetylene (Prior and Dalton, 1985).

1.18. Marine methanotrophs

The first report of the isolation of methanotrophs from the marine environment was by Hutton and ZoBell (1949). Using aged seawater or mineral salts medium they reported the enrichment of methanotrophs from a large number of different coastal mud samples. Pure cultures were obtained, but no detailed characterisations were undertaken. Heyer *et al.*, (1984) reported Type I and Type II marine methanotrophs, but no characteristics of these organisms were given. The first detailed report of the isolation and characterisation of marine methanotrophs was by Seiburth *et al.*, (1987) who described *Methylobacterium pelagicum* (*Methylobacter pelagicus*) from the upper mixing layer of the Sargasso Sea. 23 different enrichments yielded the same non-pigmented obligate Type I methanotroph, which required NaCl for growth. It only grew on methane or methanol and was inhibited by sunlight. Lidstrom (1988) has also described the isolation and characterisation of marine methanotrophs which exhibited properties of Type I methanotrophs, but contained enzyme activities of both the ribulose monophosphate pathway and the serine cycle for formaldehyde fixation. Kimura *et al.*, (1999) isolated two marine methanotrophs from coastal sediments around Gokasyo Bay in Mie Prefecture and the estuaries of Iriomote Island in Okinawa Prefecture, Japan. These have both been identified as being part of the

Methylobacterium genus. Both strains very closely resembled *Methylobacterium pelagicum*.

1.19. Environmental limits

1.19.1. Temperature

Most extant methanotrophs are mesophiles though some psychrophilic and thermophilic methanotrophs have been isolated and characterised. *Methylococcus capsulatus* is able to grow at up to 50°C and ‘*Methylothermus*’ sp. HB which was isolated from a hot spring (Bodrossy *et al.*, 1999) has a growth temperature of up to 72°C. Omel’chenko *et al.* (1992) isolated methanotrophs that had a growth optimum at 10°C or lower, three strains were grown and isolated at 3.5°C and one of these failed to grow above 15°C.

1.19.2. pH

There is relatively little known about the effect of pH on methane oxidation compared to the other factors discussed in this section. Amaral *et al.* (1998) studied several soil slurries at different pH values and demonstrated a pH optimum for methane oxidation at or near the natural soil pH. This result suggests that methanotrophs in these soils are at least partially adapted to environmental pH values. An interesting finding was that organisms extracted from soils had a narrower pH optimum range for CH₄ oxidation than the soils that they had originated from. Bacteria extracted from different soils had very similar pH responses with an optimum of 5.8-5.9, independent of which soil they originated from (Amaral *et al.*, 1998). It is a possibility that the soil matrix protects the CH₄ oxidisers from

detrimental pH values. Within the marine environment, however, very little is known.

1.20. Molecular techniques

Alternative methods which do not rely on bacterial growth on artificial media are required for the investigation of certain unique microbial communities such as the bacterioneuston.

Microbial ecology is defined as the interaction of microbes with their physiochemical environment and with other micro and macro-organisms present there. The central dogma which affects microbial ecology is the fact that only a very small proportion of the bacteria that can be visualised by direct count procedures can actually be cultivated (Ward *et al.*, 1992; Fuhrman *et al.*, 1993; DeLong, 1997). Conventional cultivation techniques incorporate biases which can mean that the few cultivatable organisms in an environment are mistaken for the most prevalent or most active. Advances in molecular biology techniques and microbial phylogeny have enabled many organisms to be identified without cultivation (Head *et al.*, 1998). Many of these techniques, including DNA extraction, PCR, cloning, sequencing, and denaturing gradient gel electrophoresis (DGGE) were used in this study.

The 16S rRNA gene occurs in all bacteria and it contains both highly conserved sequences and rapidly evolving, highly variable regions. These sequence elements, evolving at different rates, provide sufficient information to allow the measurement of both close and distant phylogenetic relationships and thus the elucidation of diversity in bacterial communities (Rochelle *et al.*, 1995).

Our perspective on microbial diversity has improved enormously over the past few decades (Hugenholtz *et al.*, 1998). This has been due, in large part, to

molecular phylogenetic studies that objectively relate organisms. Phylogenetic trees based on gene sequences are maps with which to articulate the elusive concept of biodiversity.

Based on rRNA trees, the main extent of Earth's biodiversity is microbial. Knowledge of the extent and character of microbial diversity has been limited by reliance on the study of cultivated microorganisms. It is estimated that >99% of microorganisms observable in nature typically are not cultivated by using standard techniques (Amann *et al*, 1995).

This section deals with the advantages and pitfalls of using these techniques, and whilst great advancements have been made by the expansion of molecular biology, it is important to recognise that problems and limitations do exist.

1.20.1. DNA extraction

The majority of molecular techniques used in environmental ecological studies require nucleic acids to be extracted and purified from the samples of interest. There is always an argument that if the total amount of nucleic acids present in a sample is not known then it is difficult to assess the efficiency of recovery by any extraction technique. There are many published DNA extraction methods from a variety of different environments (Ogram *et al.*, 1987; Tsai and Olsen, 1991; Jacobsen and Rasmussen, 1992). Most methods have varying combinations of physical and chemical treatments, including freeze thawing, bead-beating, lysozyme and detergent treatments. However, it is conceivable that an extraction technique may give different nucleic acid recovery efficiencies with different sample types i.e. water, soil or sediments. Ideally the degree of lysis needed for a given sample should be determined independently, as it is likely to vary between sample types. It should

be noted that spores and Gram-positive cells are more resistant to lysis than Gram-negative cells, while smaller cells (0.3-1.2 μ m) are similarly more resistant to lysis (Leff *et al.*, 1995). This has repercussions for recovery of sequences from environmental samples, where many cells may be in a state of starvation and are hence likely to form spores or reduce cell size. Another concern is the elimination of contamination of a sample during nucleic acid extraction as this may have major repercussions on further analysis and conclusions made about bacterial communities.

Leff *et al.*, (1995) compared three different DNA extraction techniques:

- 1) Ogram *et al.* (1987), which used a bead beater to disrupt cells,
- 2) Tsai and Olson (1991), where lysozyme and freeze thawing cycles were used to lyse cells,
- 3) Jacobsen and Rasmussen (1992), which employs an indirect lysis approach using cation exchange resin and lysozyme treatment.

Leff and co-workers found that all three methods had different advantages and disadvantages. For example; method 1 yielded a high concentration of DNA but it was badly sheared. Method 2 had the highest fraction of eubacterial DNA but was of lower purity. Whilst method 3 had the lowest concentrations of contaminants, but could have problems in differential recovery of cells depending on the strength of attachment to the soil or sediment matrix.

1.20.2. Polymerase chain reaction and cloning

Polymerase chain reaction amplification of nucleic acids from aquatic environments is an important tool to study the taxonomy, species diversity, distribution, occurrence, community structure, and seasonal variation of microorganisms.

The polymerase chain reaction (PCR) (Saiki *et al.*, 1985, Mullis & Faloona 1987) has revolutionised microbial ecology, allowing the amplification and analysis of bacterial DNA sequences. Moreover, in microbial community analysis and microbial interactions in aquatic ecosystems and biofilms, non-selective media may actually be selective for organisms that are eliminated during initial culture methods (Bej, 1995), thus not describing the full assemblage as these media are only selective and non-selective for known organisms. PCR primers can be designed to target conserved regions of 16S rDNA or functional genes and used to detect the presence of specific organisms, groups of organisms or functional genes in environmental samples. It is highly specific and analysis is rapid.

The use of PCR is very advantageous when limited amounts of DNA are available since the technique is both highly specific and sensitive. In this study, PCR is used for the detection of 16S rDNA sequences using universal Eubacterial primers (Chapter 2). The technique is also used for the detection of methanotrophs with primers designed for functional genes (Chapter 2).

Despite the advantages of PCR, concern has been expressed about selectivity and bias in amplification. Small differences in universally conserved regions may result in selective amplification of some sequences, especially when annealing stringency (temperature) of the primers is high. There is also a problem that more abundant sequences are preferentially amplified while low abundance sequences may be discriminated against (Farrelly *et al.*, 1995).

A study by Suzuki and Giovannoni (1996) showed that in some cases, primer bias can have an effect on PCR products generated. Generally, studies have demonstrated that when large numbers of different templates are present at low concentrations (such as in environmental samples) it is unlikely that any single

template would be present in high enough abundance to result in preferential template annealing becoming a problem.

Polz and Cavanaugh (1998) found that PCR bias may be caused by differences in G-C content at degenerate positions in primer target sites of 16S rDNA and may not be due to gene and genome dosing effects. With G-C forming a triple hydrogen bond, the melting temperatures are theoretically about 2°C higher than an A-T bond, so at each annealing step a greater proportion of templates containing G-C compliments in the binding region should hybridise to their matched primers. An alternative explanation is that AT containing primers could be more effective in forming mismatched hybrids. Little has been done to assess the presence and cause of cloning bias though it has been found that from the same batch of PCR products cloned with either sticky or blunt end cloning procedures different results were obtained (Rainey *et al.*, 1994).

The main problems highlighted here mean that compositions of clone libraries generated by PCR are a guideline and not an exact reflection of the composition of an environmental sample. A multi-aspect approach is taken in most cases to overcome the possible effects of PCR bias. This means that many different techniques should be used to study bacterial populations in environmental samples, including DGGE or SSCP (single strand complementation polymorphism), and culture studies, not just PCR and cloning.

1.20.3. Denaturing Gradient Gel Electrophoresis

Denaturing gradient gel electrophoresis (DGGE) is a relatively recently developed technique which allows PCR amplified DNA fragments of the same size to be separated by electrophoresis based on their G+C content. This method has

been successfully used on 16S rDNA PCR products from different environmental samples (Muyzer *et al.*, 1998) to directly identify the presence and abundance of different species. Banding patterns provide a profile of the population in that the relative intensity of each band and its position most likely represents the relative abundance of a particular species in the population. DGGE is rapid to perform and many samples can be run simultaneously, while further analysis of the gel is easily performed. Bands can be excised, reamplified and sequenced with relative ease and gels can also be blotted and probed for the presence of specific sequences. The limitations for DGGE are that some organisms, even though differing in G+C content, share the same electrophoretic mobility and so appear at the same position on the gel. Many organisms also show multiple banding patterns which makes the assignment of bands to specific groups of organisms difficult. Another problem is the influence of PCR bias since an initial PCR is performed and the products from this are separated on the gel. The banding pattern of a DGGE gel can only give an indication of the major species in a sample and cannot be taken as an accurate genetic fingerprint. Several studies have used DGGE to successfully target 16S rDNA sequences (Muyzer *et al.*, 1993; Ferris *et al.*, 1996; Rolleke *et al.*, 1996; Øvreas *et al.*, 1997) and functional gene sequences (Wawer and Muyzer, 1995; Rosado *et al.*, 1998; Dunfield *et al.*, 1999) in different environmental samples.

1.21. Summary

Bacterioneuston populations both consume and produce a wide variety of organic substances and as Liss (1975) suggests: "The existence of processes of this type, coupled with the high micro-organism density at the sea surface argue for the

importance of biological activity in the microlayer in bringing about the transformation of both natural and man-made materials in the marine environment”.

It has been shown, for example, that microorganisms in the ocean surface water are able to consume atmospheric CO (Conrad and Seiler, 1982; Conrad *et al.*, 1982; Conrad and Seiler, 1988). Even if the reaction rates of the bacterioneuston are too slow to interact with the gases diffusing through the surface boundary layer, they may be sufficient to consume enough from the gas phase to result in a significant gas flux from the atmosphere into the bacterioneuston layer. Neglecting this gross flux will result in overestimation of the net flux from the supersaturated surface water into the atmosphere, if the net flux is as usually calculated from transfer velocities that have been determined for biologically inert or less reactive gases, for example, SF₆ (Conrad and Seiler, 1988).

1.22. Aims and objectives

This project was carried out in conjunction with the Prof. NJP Owens, Dr. RC Upstill-Goddard and DR. T Frost from the Department of Oceanography, University of Newcastle upon Tyne, sponsored by the NERC.

The specific objectives were:

1. to determine the community structure of the bacterioneuston and bacterioplankton by comparison of 16SrRNA gene libraries of these two populations.
2. to measure invasive and evasive sea-air exchange rates of CH₄ in strictly controlled experiments in laboratory tank apparatus, thereby monitor the transfer velocity

3. to investigate the role of the microbiota in both evasive and invasive gas exchange in the experiments
4. to carryout the measurements detailed in 1 and 2 in a field situation by means of a floating box gas exchange experiments.
5. to investigate the possibility of methanotrophs within the bacterioneuston

This project focuses on a problem central to current debates about the air-sea exchange of trace gases. This study investigates, for the first time, directly the role of the sea surface microbiota in the transfer of trace gases across the sea surface interface.

Chapter Two

Materials and Methods

2.1. Sample site

All samples were taken from a site located approximately ten kilometres off the coast of Northumbria in the North Sea ($\sim 55^{\circ}08'N$ $01^{\circ}16'W$). Samples were taken in April 1998, August 1999, and April 2000.

2.2. Sea Surface Microlayer sampling

Using a sampling technique described by Norkrans (1980), a 47 mm polycarbonate membrane (Poretic Products, $2.0\ \mu m$) was placed onto the surface of the water for 10 seconds (in calm conditions). This was then removed, placed into a sterile plastic bag containing 5 ml of (see section 2.9) lysis buffer and stored at $-80\ ^{\circ}C$ until use. DNA was extracted from the membranes (method described in 2.10) and stored at $-80\ ^{\circ}C$. DNA collected was then pooled for each sample date.

2.3. Bulk water sampling

Pelagic water samples were collected from the same site by pumping water up from over 1 m below the surface into sterile gas tight bottles and kept on ice for 24hr to return to the laboratory. 500 ml aliquots of this water was then filtered through membranes ($0.2\ \mu m$, Poretic Products) and stored at $-80\ ^{\circ}C$ in lysis buffer (2.9). The DNA was then extracted from the filters (method described in 2.10).

2.4. Bacterial strains

All bacterial strains used in this study are listed in Table 2.1.

Table 2.1. Bacterial strains

Strain name	Characteristics	Phylogenetic group
Methanotrophs		
<i>Methylosinus trichosporium</i> OB3b	PMMO ⁺ /sMMO ⁺	alpha subdivision
<i>Methylocystis parvus</i> OBBP	PMMO ⁺ /sMMO ⁺	alpha subdivision
<i>Methylococcus capsulatus</i> (Bath)	PMMO ⁺ /sMMO ⁺	gamma subdivision
<i>Methylomicrobium album</i> BG8	PMMO ⁺ /sMMO ⁻	gamma subdivision
<i>Methylomonas methanica</i> S1	PMMO ⁺ /sMMO ⁻	gamma subdivision
<i>Methylomonas rubrum</i>	PMMO ⁺ /sMMO ⁻	gamma subdivision
Other organisms	Reference	
<i>Escherichia coli</i> INVαF'	Invitrogen (San Diego)	gamma subdivision
9232	Kenji, 1998	
07061	Kenji, 1998	
0-4	Kenji, 1998	
0-12	Kenji, 1998	
YO	Fuse <i>et al.</i> , 1998	
KO	Fuse <i>et al.</i> , 1998	
NI	Fuse <i>et al.</i> , 1998	

2.5. Media

All media were made up with distilled water. The pH of media was measured, corrected by the addition of 1 M HCl or 1 M NaOH and sterilised by autoclaving at 121 °C for 15 min.

2.5.1. Medium for culture and maintenance of *Escherichia coli*

Luria Bertini medium (LB) was routinely used for culturing *E. coli*. The recipe for this medium is detailed in Sambrook *et al.*, (1989).

2.5.2. Long term storage of *E.coli*.

Glycerol (75 µl) was added to 425 µl of a fresh overnight culture of *E. coli*, mixed well and then rapidly frozen in a dry ice-ethanol bath. These glycerol stocks were stored at -80°C . To recover the strain, 20 µl of the glycerol stock was used to inoculate LB media. To store large numbers of strains, glycerol stocks were prepared in 96 well microtitre plates. Glycerol was again added to a final concentration of 15% (v/v) and mixed into the culture thoroughly before storage of the microtitre plates in sealed Tupperware boxes at -20°C .

2.5.3. Media for culture and maintenance of methanotrophs

The basic mineral salts medium (Dalton and Whittenbury, 1976) was used throughout study for the routine growth of methanotrophs.

10X MS

MgSO ₄ .7H ₂ O	10 g l ⁻¹
CaCl ₂ .2H ₂ O	2 g l ⁻¹
Trace elements solution	10 ml l ⁻¹
Fe EDTA	1 ml l ⁻¹
Sodium molybdate	5 ml l ⁻¹

Trace elements solution

CuSO ₄ .5H ₂ O	0.2 g l ⁻¹
FeSO ₄ .7H ₂ O	0.5 g l ⁻¹

ZnSO ₄ .7H ₂ O	0.4 g l ⁻¹
H ₃ BO ₃	0.015 g l ⁻¹
COCl ₃ .6H ₂ O	0.05 g l ⁻¹
EDTA	0.25 g l ⁻¹
MnCl ₂ . 4H ₂ O	0.02 g l ⁻¹
NiCl ₂ .6H ₂ O	0.01 g l ⁻¹

The medium was either supplemented with 10 g l⁻¹ potassium nitrate, giving nitrate mineral salts (NMS), or with 10 g l⁻¹ ammonium nitrate, giving ammonium mineral salts (AMS). In some cases, 5 g l⁻¹ of both potassium nitrate and ammonium nitrate was added to give ammonium nitrate mineral salts (ANMS). This medium was used at a concentration of 1X for the growth of methanotrophs in liquid and on plates. For growth of pure cultures on solid media, 15 g l⁻¹ Bacto agar (Difco) was added to the basic mineral salts medium. For growth of enrichment cultures on solid media 15 g l⁻¹ Noble agar (Difco) was added to the liquid medium. Sterile phosphate solution was added to the sterile mineral salts medium when cool. For “sloppy” agars 4 g l⁻¹ Nobel agar (Difco) was added to the liquid medium.

100X Phosphate Buffer (pH6.8)

KH ₂ PO ₄	26 g l ⁻¹
NaHPO ₄ .12H ₂ O	71.6 g l ⁻¹

1 ml of phosphate buffer was added to 99 ml of 1X mineral salts medium.

2.5.4. Media for culture and maintenance of marine methanotrophs

Medium IM-310 (NANBA Kenji, personal communication)

Salts

NaCl	0.4 M
MgSO ₄ .7H ₂ O	5.0 mM

KCl	7.0 mM
CaCl ₂ .2H ₂ O	6.0 mM
KNO ₃	5.0 mM
NaHPO ₄	0.1 mM
H ₃ BO ₃	0.4 mM

Metals

EDTA-2Na ₂ .2H ₂ O	14 µM
FeCl ₂ .4H ₂ O	7.5 µM
ZnCl ₂	0.5 µM
MnCl ₂ .4H ₂ O	0.5 µM
CoCl ₂ .6H ₂ O	0.8 µM
CuCl ₂ .2H ₂ O	0.1 µM
NiCl ₂ .6H ₂ O	0.1 µM
Na ₂ Mo ₄ .2H ₂ O	0.15 µM

Buffer

NaHCO ₃	10 mM
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Basal sea water medium (Fuse *et al*, 1998)

NH ₄ NO ₃	100 mg l ⁻¹
KH ₂ PO ₄	10 mg l ⁻¹
FeEDTA	2.5 mg l ⁻¹
Vitamin B12	2.75 mg l ⁻¹
Biotin	2.5 µg l ⁻¹
Thiamine-HCl	500 µg l ⁻¹
Na ₂ EDTA	372 µg l ⁻¹
CuSO ₄ .5H ₂ O	0.25 µg l ⁻¹
ZnSO ₄ .7H ₂ O	5.75 µg l ⁻¹
MnCl ₂ .4H ₂ O	4.55 µg l ⁻¹
CoCl ₂ .6H ₂ O	0.6 µg l ⁻¹
(NH ₄) ₆ Mo ₇ O ₂₄ .4H ₂ O	0.27 g l ⁻¹

Made up in filtered seawater, pH 8.1.

2.6. Methane supplies

The methane gas used for routine culturing of methanotrophs during this study was supplied by Linde and contained 5% CO₂. The methane used for the enrichment at 20 ppm concentrations was also supplied by Linde and contained 20 ppm methane in air, although it did not contain 5% CO₂.

2.7. Maintenance and growth of methanotrophic cultures

2.7.1. Maintenance of methanotrophic cultures

Cultures were maintained by sub- culturing every 2 weeks on NMS agar plates, except for strains *Methylomonas methanica* S1 and *Methylomonas rubrum*, which were subcultured every week. The plates were incubated in 8.5 litre “Tupperware” containers fitted with airtight lids. Prior to sealing the lid, methane was introduced into the container by means of a football bladder inflated with methane to give a final concentration of approximately 20% (v/v) with air. The containers were incubated at 45 °C for *Methylococcus capsulatus* (Bath), and 30 °C for all the other methanotrophs.

2.7.2. Maintenance of marine methanotrophic cultures

Cultures were maintained by sub-culturing every three weeks into fresh media and methane added to a final concentration of 10% (v/v).

2.7.3. Isolation of methanotrophs

Dilutions (10^{-3} - 10^{-8}) of enrichments maintained at 20 ppm methane were made and 50 μ l of the dilution spread on to 1x ANMS plates. The plates were stored at room temperature in anaerobic jars containing 20 ppm methane for 2 weeks. Individual colonies, which had grown on the plates, were picked off and transferred by streaking onto fresh ANMS plates. Subsequent streaking from single colonies was performed to obtain pure isolates that were utilising methane as a sole carbon and energy source.

2.7.4. Purification of methanotrophs

The isolates obtained were checked for purity using criteria outlined by Whittenbury and Dalton (1981). Isolates were considered to be pure if colonies were similar in appearance, consisted of morphologically similar organisms, failed to grow on nutrient agar and other complex media and grew on NMS or AMS media only when CH_4 was present.

2.8. Methane measurement by gas chromatography

Methane concentrations were measured using a GCD Chromatograph (PYE Unicam) containing a 90 cm, internal diameter 4 mm, Poropak Q column (Waters, USA). Temperatures employed were; column 55 °C; injector 100 °C; detector 200 °C. The range was set to 1 x 10. Data from the GC were recorded on a 3390A integrator (Hewlett Packard) which had set parameters as follows;

Attenuation	-1
Peak width	0.10

Threshold 1

Area to reject under 1000

Standard samples were used to calibrate the GC before each use. 500 µl of sample was introduced to the GC with a 1 ml syringe (Becton Dickinson) fitted with a 25 gauge needle (Becton Dickinson).

2.9. Buffers, reagents and solutions

2.9.1. General purpose buffers

Solution/reagent	Constituent(s) per litre H ₂ O
TE Buffer	10 mM Tris base 50 mM EDTA Adjust to pH 8.0
SET Buffer	Sucrose 200 g 0.5 M di-sodium EDTA 100 ml 1 M Tris HCl (pH 7.6) 50 ml
20X Standard saline citrate (SSC)	Sodium chloride 175.3 g Tri-sodium citrate 88.2 g
Lysis Buffer	20 mM EDTA 400 mM NaCl 0.75 M Sucrose 50 mM Tris HCl pH 9.0

2.9.2. Electrophoresis solutions/buffers

40% acrylamide (Phigel 3 from Fischer)	Acrylamide/ Methylene-bisacrylamide 40% solution
50X TBE	Tris base 108 g Boric acid 55 g 0.5 M EDTA (pH8) 40 ml Make up to 1 litre with distilled water.
50X TAE	Tris base 242 g Glacial acetic acid 57.1 ml 0.5M EDTA (pH 8.0) 100 ml Make up to 1 litre with distilled water.
Agarose gel loading buffer (6X)	Bromophenol blue 0.0125 g Ficoll (type 400) 0.75 g Distilled water 5 ml
0% denaturant acrylamide stock make up to 100 ml with H ₂ O	40% acrylamide 25 ml 50X TAE 2 ml
100% denaturant acrylamide stock make up to 100 ml with H ₂ O	40% acrylamide 25 ml 50X TAE 2 ml Formamide 40 ml urea 42 g

2.9.3. Silver staining solutions

Ethanol/acetic acid	Ethanol	10% v/v
	Acetic acid	0.5% v/v
Staining solution	Silver nitrate	0.1% w/v
Developing solution	NaOH	1.5% w/v
	NaBH ₄	0.01% w/v
	Formaldehyde	0.15% v/v
Fixation solution	Na ₂ CO ₃	0.75% w/v

2.9.4. Buffers and solutions for plasmid extraction from *E. coli*

	Constituent(s) per 250 ml H ₂ O	
Solution I	Glucose	2.25 g
	Tris base	0.76 g
	Di-sodium EDTA 0.5 M	5 ml
Solution II	10 M NaOH	5 ml
	10% (w/v) SDS	25 ml
Solution III	Potassium acetate	73.6 g
	Glacial acetic acid	30 ml

2.9.5. Solutions and reagents for hybridisation

	Constituents.
Denaturing solution 1 L	121.14 g Tris-HCl 87.66 g NaCl pH 8.0
Neutralising solution 1 L	87.66 g NaCl 20 g NaOH
Prehybridisation solution 50 ml	12.5 ml SSC (20 x) 2.5 ml 10% (w/v) SDS 5 ml Denhardts (50 x)
Denhardts (50x)	10 g Ficoll 400 10 g Polyvinylpyrrolidone 360 10 g Bovine serum albumin (Sigma)

2.10. DNA extraction and purification

2.10.1. Precipitation of nucleic acids

0.1 volumes of 3 M sodium acetate and 3 volumes of ethanol were added to the solution containing nucleic acid and incubated for at least 2 hr at -20°C . Nucleic acids were collected by centrifugation for 20 min at 11,600 g and 4°C . The pellet was washed in 70% (v/v) ethanol and air dried before resuspension in an appropriate volume of sterile TE buffer or sterile distilled water.

2.10.2. DNA extraction from filters

A modification of the method from Giovannoni *et al.*, (1990) was used for DNA extraction from all filters.

Total DNA was extracted from the membrane samples, both sea surface microlayer and pelagic layers. The extraction method consisted of adding SDS to 1% (v/v), proteinase K to 100 µg/ml and incubating the bag containing the membrane on a moving platform at 37 °C for 30 min. The bag and contents were then heated to 55 °C for 10 min with continued agitation.

The liquid fraction was then transferred to a Corex tube, 1 volume phenol chloroform iso amyl alcohol added and centrifuged for 5 min at 2,800 xg. The aqueous phase was transferred to a clean tube and after precipitation at -20 °C overnight with 0.1 x volumes of 2 M sodium acetate and 2 volumes of 100% ethanol centrifuged at 5,600 xg for 30 min, the resultant pellet was resuspended in 300 µl TE.

2.10.3. Extraction of DNA from enrichment cultures

DNA extraction from the enrichments used a method from Marmur (1961).

2.10.4. Extraction of DNA from pure methanotroph cultures

Methanotroph DNAs were prepared by the method described by Oakley and Murrell (1988).

2.10.5. Plasmid extraction from *E. coli*

The method of Saunders and Burke (1990) was used to purify plasmids from *E. coli*.

2.10.6. Quantification of DNA

Spectrophotometer readings were taken at an absorbance of 260 nm for solutions containing nucleic acids using quartz cuvettes in a Beckman DU-70 spectrophotometer. At A_{260} nm, a reading of 1.0 was taken to be equivalent to 50 mg ml⁻¹ of DNA (Sambrook *et al.*, 1989).

2.11. Restriction endonuclease digestion of DNA

GIBCO BRL supplied restriction enzymes and buffer solutions. The DNA was digested according to the manufacturer's instructions.

2.11.1. Restriction fragment length polymorphism (RFLP)

Plasmids containing amplified PCR products were isolated from clones using the method described in 2.10. They were then digested with different sets of enzymes, *RsaI* and *EcoRI* in reaction buffer 4 for three hours and *PvuI*, *HincII* and *EcoRI* in reaction buffer 6 overnight. Restriction fragments were separated using agarose gel electrophoresis (Section 2.8.9). A 2% (w/v) agarose gel was used to resolve DNA fragments of 50-100 bp.

2.12. Agarose gel electrophoresis

Agarose (Gibco BRL) gels were made and run in 0.5 X TBE or 1 X TAE buffer. Routine analysis of DNA samples were done on 1% (w/v) agarose gels. Gels for the RFLP analysis contained 2% (w/v) agarose to resolve smaller DNA bands. Ethidium bromide (EtBr) was added to the gel at a final concentration of 0.5 µg / ml. Mini-gels were run at a constant current of 55 mV and DNA was visualised by

placing the gel on a UV transilluminator. Gel images were recorded with either a Polaroid camera (CU5 Land Camera, loaded with 665 positive/negative film) or a gel documentation system (UVP life sciences Grab It 2.0 Synoptics Ltd.).

2.12.1. Elution of DNA from agarose gels

DNA fragments were excised from agarose gels and then eluted using the GeneClean II kit (Bio 101 California, USA) according to the manufacturers instructions.

2.13. Polymerase Chain Reaction

Polymerase chain reaction (PCR) was used to detect and amplify genes of interest from total soil DNA and bacterial isolates. Primers used in this study are listed together with reaction conditions, in Table 2.2.

2.13.1. Standard PCR reaction

PCR amplification was performed in a total volume of 50 µl in a 0.5 ml microfuge tube. Each tube contained 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris (pH9.0), 100 µl each dNTP and 50 pmol of each primer. Template DNA at a concentration of 1ng was added to the reaction mix. Samples were placed into a PCR machine (Touchdown thermal cycling system, Hybaid). The reaction mix underwent a “hot start” where the temperature was increased to 94°C for 5 min followed by a temperature decrease to a specifically chosen temperature for 1 min. At this stage, 2.5 units of *Taq* polymerase (Gibco BRL) was added and the reaction continued. The reaction conditions were 94 °C for 1 min (denaturation), variable

Table 2.2. Primers and annealing temperatures used for PCR in this study

N= A:G:C:T S=G:C K=G:T Y=C:T R=A:G M=XXXX f=forward r=reverse

Primer name	Sequence 5' ————— 3'	Target gene	Target groups	Annealing temperature °C and time in seconds	Reference
f27	AGAGTTTGATCMTG GCTCAG	16S rDNA	most eubacteria	60/60	Lane, 1991
1492r	TACGGYTACCTTGTT ACGACTT	16S rDNA	most eubacteria	60/60	Lane, 1991
907r	CCCCTCAATTCCTTT GAGTTT	16S rDNA	most eubacteria, archaea.	60/60	Teske <i>et al.</i> , 1996
357f	CTCCTACGGGAGGC AGCAG	16S rDNA	most eubacteria	60/60	Lane, 1991
<i>mx</i> <i>aF</i> 1 1003f	GCGGCACCAACTGG GGCTGGT	<i>mx</i> <i>aF</i> (methanol dehydrogenase)	Gram negative methylootrophs	59/60	McDonald <i>et al.</i> , 1995
<i>Mx</i> <i>aF</i> 2 1561r	GGGCAGCATGAAGG GCTCCC	<i>mx</i> <i>aF</i> (methanol dehydrogenase)	Gram negative methylootrophs	59/60	McDonald <i>et al.</i> , 1995
<i>m</i> <i>mo</i> <i>X</i> 1 882f	GGCTCCAAGTTCAAG GTCGAGC	<i>m</i> <i>mo</i> <i>X</i> (soluble methane monooxygenase)	sMMO ⁺ methanotrophs	55/60	McDonald <i>et al.</i> , 1995

<i>mmoX</i> 2 1403r	TGGCACTCGTAGCGC TCCGGCTCG	<i>mmoX</i> (soluble methane monooxygenase)	sMMO ⁺ methanotrophs	55/60	McDonald <i>et al.</i> , 1995
X536f	CGCTGTGGAAGGGC ATGAAGCG	<i>mmoX</i> (soluble methane monooxygenase)	sMMO ⁺ methanotrophs	65/60	Fuse <i>et al.</i> , 1998
X898r	GCTCGACCTTGAAC TGGAGCC	<i>mmoX</i> (soluble methane monooxygenase)	sMMO ⁺ methanotrophs	65/60	Fuse <i>et al.</i> , 1998
<i>pmoA</i> 189f	GGNGACTGGGACTT CTGG	<i>pmoA</i> and <i>amoA</i> (particulate methane and ammonia monooxygenase)	methane and ammonia oxidisers	56/60	Holmes <i>et al.</i> , 1995
<i>pmoA</i> 682r	GAASGCNGAGAAGA ASGC	<i>pmoA</i> and <i>amoA</i> (particulate methane and ammonia monooxygenase)	methane and ammonia oxidisers	56/60	Holmes <i>et al.</i> , 1995
mb661	CCGGMGCAACGTCY TTACC	<i>pmoA</i> (particulate methane monooxygenase)	methane oxidisers	55/60	Costello & Lidstrom, 1999.
GC-GM5f	CGCCCGCCGCGCCCC GCGCCCGTCCCGCCG CCCCCGCCCGCCTAC GGGAGGCAGCAG	16S rDNA	Most eubacteria	Touchdown reaction. Reduced by 0.5°C from 70-60°C over 20 cycles then 10 cycles at 60°C	Teske <i>et al.</i> , 1996

temperature according to primers used for 1 min (annealing) and 72 °C for 1 min (extension). These conditions were cycled 30 times and a final extension step of 72 °C for 5 min completed the PCR.

2.14. Cloning of PCR products

Taq polymerase has no 3' to 5' exonuclease activity and therefore a 3' adenine overhang remains on the ends of the PCR products. These were cloned using the TA cloning Kit or the TOPO cloning Kit (Invitrogen, San Diego, CA) according to the instructions of the manufacturer.

2.15. DNA sequencing

Sequence information was obtained using an Applied Biosystems Model 373A automatic sequencer (run by the Departmental Molecular Biology Central Facility). Sequencing reactions were carried out by cycle sequencing with the Dye Terminator Kit from PE Applied Biosystems. Cycle sequencing (25 cycles) was performed in a PCR machine (Touchdown thermal cycling system, Hybaid) with conditions,

96 °C for 30 seconds

50 °C for 15 seconds

60 °C for 4 minutes

2.16. Sequence analysis

Sequences were edited using the Chromas sequence analysis package (McCarthy, Griffith University, Australia). Analyses of DNA sequences were performed using the Basic Logic Alignment Search Tool (BLAST) programme, which is found on the World Wide Web <http://www.ncbi.nlm.nih.gov/> to make preliminary phylogenetic inferences about relatedness. Sequences were aligned using a sequence editor against reference sequences and analysed using the ARB software package (Strunk and Ludwig, 1996).

The ARB programme (<http://www.mikro.biologie.tu-muenchen.de>) was used for sequence alignment and phylogenetic analyses. All 16S rDNA sequences were aligned using the automatic alignment tool of ARB (ALIGNER V2.0) and corrected manually according to secondary structural constraints. Aligned sequences were added to a tree of sequences (>1400 bp) with a maximum parsimony tool within ARB. A filter was generated that omitted alignment positions of sequence ambiguity (N) and where sequence data were not available for all near full length sequences.

In order to evaluate the tree topology, phylogenies were reconstructed with various data subsets using evolutionary distance (Jukes and Cantor model), maximum parsimony (ARB and DNAPARS) and maximum likelihood (default parameters for ARB and fastDNAmI) analyses of the aligned sequences.

Functional gene sequences were aligned manually to related sequences extracted from GenBank. A filter was generated from the deduced amino acid sequences that excluded alignment positions containing amino-acid ambiguity (X) and missing sequence data. Phylogenies were reconstructed with evolutionary distance (DayhoffPAM model), maximum parsimony (ARB and PROTPARS) and maximum likelihood (default parameters for ARB and Protein-ML) analyses.

2.17. Denaturing Gradient Gel Electrophoresis

2.17.1. Addition of GC clamp

To allow DGGE analysis of PCR products, a high GC oligomer was added to specific primer pairs. The 'clamp' was only attached to one of the primers at the 5' end and the other primer was left unchanged. This clamp allows denaturation to occur, but holds the two strands together so they cannot completely separate. A touchdown PCR was used for DGGE PCR reactions (Don *et al.*, 1991). This employs an elevated annealing temperature, which then decreases successively at each cycle. This method is used to increase specificity of the primers. The PCR products were run on an agarose gel to verify size. If non-specific bands were observed, the PCR was repeated under more stringent conditions until only one band of the expected size was seen. The D-CODE universal mutation detection system (Biorad) was used for electrophoresis.

In this study DGGE analysis was performed on 16S rDNA PCR generated fragments using the primers GC-GM5f and 907r.

2.17.2. Travel Denaturing Gradient Gel Electrophoresis

Once perpendicular DGGE gels had been used to establish optimum gradients parallel gels were used to establish the optimal time for separation. Two different PCR products derived from a PCR using the same primer set were mixed together (1:1) and an equal amount of loading buffer added. Aliquots of this mix were added at 30 min time intervals for 8 hr. During this time the gel was run at 150 V in 1x TAE buffer at 60°C.

2.17.3. Parallel Denaturing Gradient Gel Electrophoresis

Once optimum conditions for the gradient and the time of the run had been determined the samples of interest were run on a parallel denaturing gel. Gels were cast according to manufacturers instructions and left overnight to polymerise. The PCR product was mixed with the loading buffer and loaded into the wells. Electrophoresis conditions were, 200 mV, 1x TAE at 60 °C for 6 hrs.

2.17.4. Perpendicular Denaturing Gradient Gel Electrophoresis

Optimal gradients for the melting of PCR products are established using perpendicular gradient gels. A 0-100% formamide denaturant gradient was set up horizontally along the gel using the model 475 gradient former according to the manufacturer's instructions (Biorad).

2.17.5. Staining of denaturing gradient gels

Three different methods were used in this study to stain DGGE gels. The first was ethidium bromide staining where the gel was stained in 100 ml of 1 x TAE buffer containing 50 µg / ml ethidium bromide. This was gently agitated for 15 min and then the solution was removed and replaced with distilled water and left for 10 min. The gel was visualised under UV transilluminator and photographed (Polaroid P665 film) or visualised using the gel documentation system (UVP life sciences Grab It 2.0 Synoptics Ltd.).

The second method of staining used 1X Syber Gold (Molecular probes, Eugene, Oregon) in 1 x TAE buffer. Syber gold stain was 25-100 x more sensitive

than ethidium bromide staining (Molecular probes Product information sheet S-11494 1987)

The third method, silver staining, involved incubating the gel twice for three minutes in the ethanol/acetic acid solution. Removing the excess solution and incubating the gel in the silver nitrate solution for 20 minutes. The gel was then rinsed thoroughly in sterile distilled water and immersed in developing solution for 30 minutes. The gel was then removed from the developer and placed in fixative for 10 minutes and then visualised on white light and recordings made.

2.17.6. Excision and sequencing of bands from DGGE gels.

Bands were excised from the gel using sterile pipette tips. The pieces of acrylamide containing the bands were incubated in 100µl of sterile dH₂O at 4 °C overnight. A 5 µl aliquot of this solution was used for PCR amplification. PCR products were re-run on the DGGE to confirm the purity of the band and the PCR product was purified with the Wizard PCR preps DNA purification system (Promega) as per manufacturers instructions. The cleaned PCR product was subjected to cycle sequencing (section 2.15)

2.18. Nucleic acid hybridisation

2.18.1. Colony blot hybridisation

Recombinant clones containing 16S rRNA amplified inserts were toothpicked into 96 well microtitre plates containing LB and antibiotics and grown over night at 37 °C. These were then replica plated onto nylon membranes (Hybond-N) lying on a LB agar plate containing antibiotics (Ampicillin and Kanamycin). These plates were

incubated at 37 °C overnight to allow growth of the clones. Four trays containing 2 sheets of 3M Whatman paper were set up and the paper wetted with the appropriate solutions. Recipes for Denaturing and Neutralising solutions are given in section 2.9.4. The membranes were placed in each tray in the order and for the length of time indicated below.

Tray1	10% (w/v) SDS	2 min
↓		
Tray 2	Denaturing solution	15 min
↓		
Tray 3	Neutralising solution	15 min
↓		
Tray4	6 x SSC	15 min

The membranes were then air dried for 30 min before the DNA was fixed using a UV “Stratalinker” (Stratagene).

2.18.2. Prehybridisation

Filters were covered with nylon mesh and placed into a Hybridisation tube. Prehybridisation solution (35 ml) was added and the tube was incubated in a Hybridisation oven at 50 °C for 3 hours.

2.18.3. Nick translation

Probe DNA was labelled with α -³²P dNTP. Nick translation was carried out as described in Sambrook *et al.* (1989). The probe was denatured by incubation at 100 °C for 10 min before hybridisation.

2.18.4. Hybridisation

The prehybridisation buffer was removed and 15 ml of fresh prehybridisation buffer were added to the filter in the tube. The radioactive probe was added and the bottle was incubated in the Hybaid oven at 50 °C overnight.

2.18.5. Stringency washes

The filter was washed with 6 x SSC at 65 °C for 2 hr to remove any unbound or non-specifically bound probe. The filters were dried and wrapped in cling film.

2.18.6. Radioactive filter stripping

The radioactive filter was placed into a Hybaid tube and stripped by adding 100 ml of boiling 0.1% (w/v) SDS. The filter was washed for 30 mins and then checked for residual radioactivity using a Geiger counter. If it was still radioactive, the procedure was repeated.

2.18.7. Autoradiography

Fuji nif RX medical X-ray film was used for autoradiography. Radioactive membranes were exposed to the film in autoradiography cassettes with intensifying

screens (Molecular Dynamics) at -70°C . The length of exposure time was dependent on signal intensity. Films were developed in appropriately diluted developer and fixer solutions (Kodak Pathe).

2.19. Microscopy

2.19.1. Light microscopy

A Kyoga-Unilux III (Tokyo) phase contrast microscope was used for routine microscopy. Microbial cultures were examined under oil immersion at 1000x magnification. Photographs of microbial cultures were produced using a Zeiss Axioskop (Zeiss, Germany) phase contrast microscope at 1000x magnification with Kodak panther 1600 film.

2.20. Statistical analysis

DNA extractions were performed in parallel and samples pooled. Gas Chromatograph measurements were performed in triplicate and standard error calculated for each point.

Chapter Three
Molecular Investigation
of the Bacterioneuston

3.1. Introduction

From the Introduction to this thesis, it is clear that bacterioplankton are recognised as important agents of biogeochemical change in marine ecosystems, yet relatively little is known about the species that make up these communities (Giovannoni *et al.*, 1990), especially those which make up the bacterioneuston.

3.1.1. The bacterioneuston

The bacterioneuston is the assemblage of bacteria found in the sea –surface microlayer. This microlayer is an important interface between the atmosphere and the hydrosphere (Hardy and Apts, 1984). It is an area which has been shown to attract atmospheric pollutants of natural and man-made origin, containing high concentrations of natural macromolecules and carrying a unique fauna.

The juxtaposition of materials and neuston at this interface allows for a highly productive open system that plays a significant role in biogeochemical cycling (Frost, 2000). However, concentration at this interface also increases the likelihood of neuston exposure to a greater variety of stresses, such as solar radiation, and daily changes in both salinity and temperature.

This microlayer covers the upper 10 to 1000 μm deep boundary layer of the ocean. In most, if not all of the investigations of this zone, the surface film is operationally defined based upon the depth of the sample collected, which is in turn, dependent on the sampler employed (Garret and Duce, 1980). In this study we have isolated the uppermost 30 μm from the sea surface and compared the bacterial assemblage found within this microlayer with that of a sample from the bulk water column at least 1 metre below.

The accumulation of high numbers of bacteria in surface films has been demonstrated in numerous reports and appears to be a typical phenomenon (Norkrans, 1980; Hardy, 1982; Kjelleberg, 1985). Although there is considerable literature regarding the numbers of organisms found within, and activity of, the bacterioneuston, there have been few detailed studies of the make up of the bacterioneuston using a molecular biological approach. Many studies have indicated that viable bacteria, as determined by colony forming units on solid media, were more numerous in the sea-surface microlayer than in the plankton (Danos *et al.*, 1983; Maki, 1993). Discrepancies between direct counts and plate counts are typically several orders of magnitude, raising doubts as to whether cultivated marine bacteria are actually representative of dominant planktonic species (Giovannoni *et al.*, 1990).

Uncertainties about the genetic structure and diversity of natural bacterioplankton populations stem from the traditional difficulties associated with microbial cultivation techniques (Giovannoni *et al.*, 1990). This has been a considerable handicap to microbial ecology. Ecological inferences based on the metabolic properties of cultivated bacteria are unrepresentative of the natural populations from which they were obtained (Head *et al.*, 1998).

3.1.2. Molecular methods

Due to the ubiquity of ribosomal RNA molecules in all cellular life forms, comparative analysis of their sequences can be universally applied to infer relationships between organisms. The rRNA molecules comprise highly conserved sequence domains interspersed with more variable regions (Gutell *et al.*, 1994). In general, essential rRNA domains are conserved across all phylogenetic domains, thus

universal tracts of sequences can be identified, and it is also possible to identify sequence motifs of increasing phylogenetic resolution. For example, short stretches of sequence characteristic of a number of the bacterial divisions and subdivisions (α -, β -, δ -, γ -Proteobacteria, high %G+C Gram-positive bacteria and the *Flavobacterium-Cytophaga* division) have been identified (Head *et al.*, 1998).

The most commonly used form of comparative rRNA sequence analysis involves the construction of phylogenetic trees. These are the product of phylogenetic analyses and are a dynamic representation of the relationships between microorganisms (Stackebrandt, 1992, O'Donnell *et al.*, 1993). The greatest value of phylogenetic analysis with 16S rRNA is in identification at the level of genus and above (Vandamme *et al.*, 1996). It can also detect lineages which, as yet, do not contain sequenced isolates and place these lineages within the existing evolutionary framework (Giovannoni *et al.*, 1990). The groups which are recovered and the accuracy of identification is determined by the quality of the sequence data, the choice of reference sequences, the regions chosen for analysis and the computational methods used to infer the phylogenies (Olsen and Woese, 1993; Vandamme *et al.*, 1996). Once appropriate sequences have been chosen and homologous nucleotide positions have been aligned, dendrograms can be assembled from the data sets.

3.2. Materials and Methods

These are detailed in Chapter 2, Materials and Methods.

Sample collection and DNA extraction was carried out as described in Chapter 2 and will also be covered later in this chapter, as development of these methods form part of the study.

3.2.1. PCR amplifications

Primers f27 and 1492r (Table 2.2) were used to target eubacterial copies of the 16S rDNA (Lane, 1991) present. These were used in PCR experiments, using the standard conditions (Table 2.2). Products of the predicted size (~1.5 kbp) were obtained from the environmental samples (section 3.3.1).

3.2.2. Construction of clone libraries

Libraries were constructed as described in Chapter 2. Over 500 colonies containing inserts were identified from both the bacterioneuston and pelagic libraries, i.e. the amplification of the DNA extracted from the sea surface microlayer and the DNA isolated from the pelagic waters collected (section 3.3.1). Another 1000 clones were picked from the libraries for use in DNA hybridisation probings (section 3.3.7).

3.2.3. Restriction digests

The initial screening of the two libraries made was by Restriction Fragment Length Polymorphisms (RFLP) using the restriction enzymes *EcoRI*, *RsaI*, *HincII* and *PvuII* (Rooney-Varga *et al.*, 1998).

3.2.4. Colony hybridisation

Using the probes Vibsp1 (TGTCGTTTCCGCTCGACT) for *Vibrio splendidus* and other *Vibrio* species designed in this study, and Psa11 (CCAGCTTCTAGTAGACATCGTT) for most *Pseudoalteromonas* species, designed for this study, targeting specific fragments of the 16S rRNA gene. The libraries were screened as described in Chapter 2. The probes were designed using

the ARB package (Strunk and Ludwig, 1998), with reference to 16S rDNA from known strains and also sequences found in this study.

3.2.5. DNA Sequencing

This was carried out as described Chapter 2. Representatives of all the major Operational Taxonomic Units (OTUs) (Moyer *et al.*, 1994) (defined by experiments in section 3.3.1) were chosen for sequencing. Plasmid DNAs from selected 16S rDNA clones were sequenced (partially, over 500 bp) by *Taq* cycle sequencing.

3.2.6. Comparative sequence analysis

New partial sequences (>500 bp) were analysed using BLAST (Altschul *et al.*, 1990) and added together with most important BLAST hits, to an alignment of about 5300 homologous bacterial 16S rRNA primary structures (Maidak *et al.*, 1997) by using the aligning tool of the ARB software package (Strunk and Ludwig, 1998). Sequences were checked for chimera formation with the CHECK_CHIMERA software of the Ribosomal Database Project (Maidak *et al.*, 1997). Potential chimeras were eliminated before phylogenetic trees were constructed section (2.16).

3.2.7. Sampling the Bacterioneuston

A number of criteria such as, cost, ease of use, storage and reproducibility dictated the sample method chosen for this study. The sampling vessel was a small two man Zodiac launched from a research vessel (*Benicia*, Newcastle University), which was deployed out of the harbour at Blythe in Northumbria. The sample area was started from 55°08'N 01°16'W, which is approximately 10 kilometres off the

coast from Blythe. The sample vessel was then allowed to drift with the tides whilst samples from the surface were taken. Due to the limited space available on the Zodiac (~250 cm by 100 cm), the sample method needed to be small enough to transport from *Benicia* to the Zodiac, be safe and easy to use on the Zodiac, easy to store whilst on the Zodiac, easy to transport back from the Zodiac to *Benicia* and then store before use in the laboratory. This meant the sample method chosen from those available and which would fulfil the majority of these criteria was fairly obvious, the 47 mm polycarbonate membrane.

Previous literature concerned with sampling the sea surface microlayer either for biological content (Norkrans, 1980) or chemical content (Hardy, 1982) has explored a number of sampling methods. Examples of the most relevant as well as their estimated sample depth are listed in Table 3.1.

3.2.7.1. Sampling method used

For the purposes of this study, we chose the membrane method (a, Table 3.1) as it sampled the depth we required, and when tested, the depth of sample was consistent over a number of membranes, and its small size lent itself easily to our criteria. A number of different filters were tested and all could have been used but the one chosen was inexpensive and consistent. The depth of sample taken was estimated by weighing five of the membranes before placing them on the surface of a bucket of water and then weighing them again. This was repeated another four times, with new membranes, to obtain a mean value for the amount of water picked up on one membrane and then the depth of the samples calculated using the formula

πr^2 . So for a 47 mm diameter membrane (surface area 1734.9 mm²) the depth sampled was ~35 μ m.

$$\pi r^2 = 3.14 \times 23.5^2 = 3.14 \times 552.25 = 17.35 \text{ cm}^2$$

This method was used for a range of membranes (differing pore size, membrane diameter, and material) the one that fitted into the study most consistently was the 47 mm diameter 2 μ m pore polycarbonate membrane made by Poretics. The main problems with the other sampling methods were the size of the equipment. They are much too big for the conditions in the Zodiac. They are hard to clean/sterilise between samples, difficult to collect sample from sampler, and some were difficult to obtain and use. Once the samples had been taken, their storage and subsequent transport from the Zodiac to the laboratory would have been the same for all the methods considered.

Once the sample method had been decided, the sample trips could be planned and executed. The major consideration for the sampling trips using the membranes was wind speed. If the wind was not calm, that is below a Force 3 (Beaufort scale), then the membrane would either blow away or the water collected would be shaken off the membrane as it was removed and before it could be stored. This dictated that for a better chance of successfully sampling, the microlayer the samples needed to be taken in fine weather and so year round sampling was not deemed a viable option.

Table 3.1. Aquatic surface microlayer sampling methods.

Sampler	Sample thickness (µm)	Samples collected	Reference:
Freezing Probe	1000	Seawater and particles	Hamilton and Clifton (1979)
Mesh Screen (Garrett Screen)	150-400	Microbiology and lipids and fatty acids	Garrett (1965), Sieburth (1965) Duce <i>et al.</i> (1972)
Rotating Drum	60-100	Microbiology and organics	Harvey (1966)
Glass Plate	20-100	Chemical and microbiological	Harvey and Burzell (1972)
Hydrophilic Nuclepore Membrane	4-40	Microbiology and organics	Crow <i>et al.</i> (1975)
Perforated Teflon Plate	100-50	Glycoproteins and other organics	Larsson <i>et al.</i> (1974) Norkrans and Sorensson (1977)
Teflon Sheet	10	Hydrocarbons and fatty acids	Kjelleberg <i>et al.</i> (1979)
Hydrophobic Nuclepore Membrane (a)	20-50	Microbiology and organics	Kjelleberg <i>et al.</i> (1979)
Bubble microtome	1	Fractionated chemical and microbiological aerosol over sea surface	MacIntyre (1968)
Germanium Prism	0.01	Organics	Baier (1972)
Skimmer Nets	100000-10000	Zooneuston	Eldridge <i>et al.</i> (1977)

3.3. Results

3.3.1. RFLP analysis of the Bacterioneuston vs. the pelagic water

Before sampling of the ocean could be carried out, the sampling method needed to be validated. To be certain all DNA collected during a sampling experiment was from the environment, all equipment and solutions were investigated to ensure these would contain no contamination. Experiments show the only source of amplifiable DNA from a filter extraction will come from something the filter has picked up and not from within the sampling or extraction methods used (Figure 3.1).



Figure 3.1. PCR amplification of 16S DNA from possible sources of contamination. Lane Order; 1 Sample Bag plus Lysis Buffer; 2 Sample Bag, Lysis Buffer plus 10% SDS; 3 Sample Bag, Lysis Buffer, 10% SDS plus Proteinase K; 4 Sample Bag, Lysis Buffer, 10% SDS, Proteinase K plus a membrane (UV); 5 Sample Bag, Lysis Buffer, 10% SDS, Proteinase K plus a membrane; 6 Sample Bag, Lysis Buffer, 10% SDS, Proteinase K plus a membrane; 7 Sample Bag, Lysis Buffer, 10% SDS, Proteinase K plus a membrane; 8 Sample Bag, Lysis Buffer, 10% SDS, Proteinase K plus a membrane placed on sterile H₂O; 9 Sample Bag, Lysis Buffer, 10% SDS, Proteinase K plus a membrane placed on sterile H₂O; 10 Control DNA extracted from *Methylosinus trichosporium* OB3b; 11 one kb DNA Ladder (molecular mass markers).

Experiments to demonstrate the ability to isolate DNA from filters, which have been laid on the sea surface microlayer, and quantify this DNA were performed (Figure 3.2). The DNA was quantified by comparison with DNA of known concentration. As described in Figure 3.1 the filter from the microlayer must have picked up this DNA.

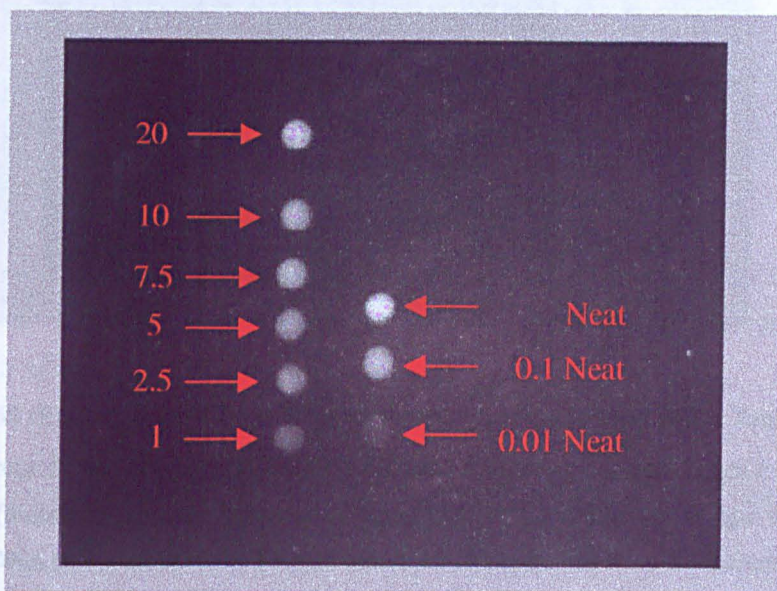


Figure 3. 2. DNA extracted from filters(neat, a 1:10 and a 1:100 dilutions of neat), which have been in contact with the bacterioneuston along side samples of DNA of known concentration. The concentration of DNA on the left hand side is in $\mu\text{g ml}^{-1}$.

We can, therefore, estimate the concentration of DNA after extraction, from 1 filter, to be approximately $20 \mu\text{g ml}^{-1}$.

The DNA extracted from the environment is amplifiable by the eubacterial primers (Figure 3.3) and therefore it is possible to infer the method must be picking up eubacterial DNA from both the pelagic water and the sea surface microlayer. Thus so far validating the choice of sampling strategy.

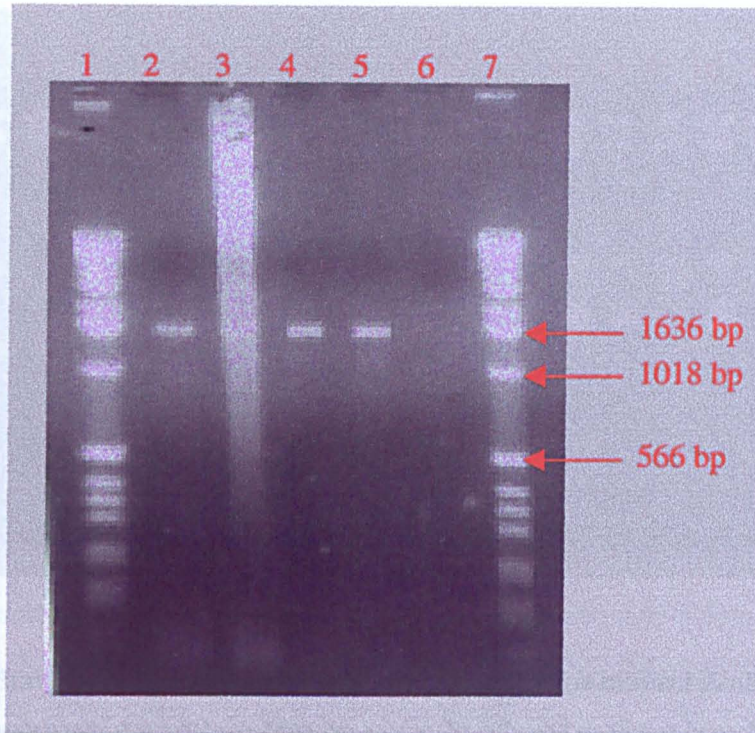


Figure 3.3. 16S rRNA PCR amplification (using the PCR primers f27 and 1492r, Table 2.2) of DNA extracted from the bacterioneuston and DNA isolated from pelagic water. Lanes 1 and 7 were 1 kb DNA Ladder (molecular mass markers); lane 2 neat DNA extracted from the Pelagic water; lane 3 1:10 dilution of DNA extracted from the Pelagic water; 4 DNA extracted from the Bacterioneuston samples taken; 5 DNA extracted from the Bacterioneuston samples taken; 6 Negative Control. Lane 3 should be discounted as it has been contaminated.

Initial investigation into the bacterial content of the sea surface microlayer involved constructing libraries of 500 16S rRNA clones (PCR primers f27 and 1492r, Table 2.2) using DNA extracted and then amplified from each habitat (sea surface and pelagic water) sampled. Clones were then chosen at random, digested with restriction enzymes to reveal RFLPs for each clone and these RFLPs were separated into OTUs.

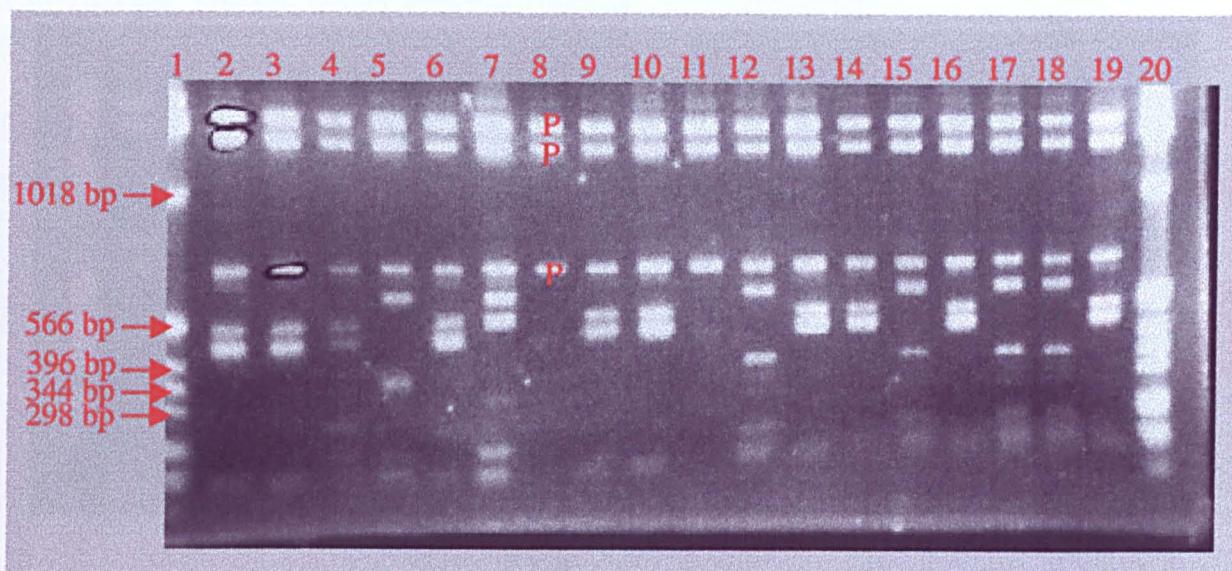


Figure 3.5. Restriction digests of 16S rRNA clones from the bacterioneuston library cut with *EcoRI* and *RsaI*.

Figure 3.4. Examples of Bacterioneuston clones cut with restriction enzymes *EcoRI* and *RsaI*.

Lane 8 shows those bands that originate from the vector (P) only and so appear in each lane. Ringed bands should be ignored

Figures 3.4, 3.5 and 3.6 represent a number of different RFLP experiments indicating that differing species are present in the bacterioneuston. *EcoRI* releases the amplified fragment from the vector so all the other bands must be from the cloned 16S rRNA fragment. Also demonstrated in these figures is the difference between the two environments, the sea surface microlayer and the pelagic water, Figures 3.4 and 3.5 are examples from the bacterioneuston and have only a few different RFLP patterns and so less OTUs. Whereas, Figure 3.6 is an example from the pelagic 16S rRNA clone library and demonstrates a greater variety of RFLPs.

From these results there was evidence of a difference between the assemblage of the bacterioneuston and that of the pelagic waters.

The random clones chosen from the 16S rRNA clone libraries were cut with *EcoRI* to release the insert and digested with *RsaI* to give a distinct RFLP. Clones were also digested with *EcoRI*, *HincII* and *PvuII* to further separate the clones into

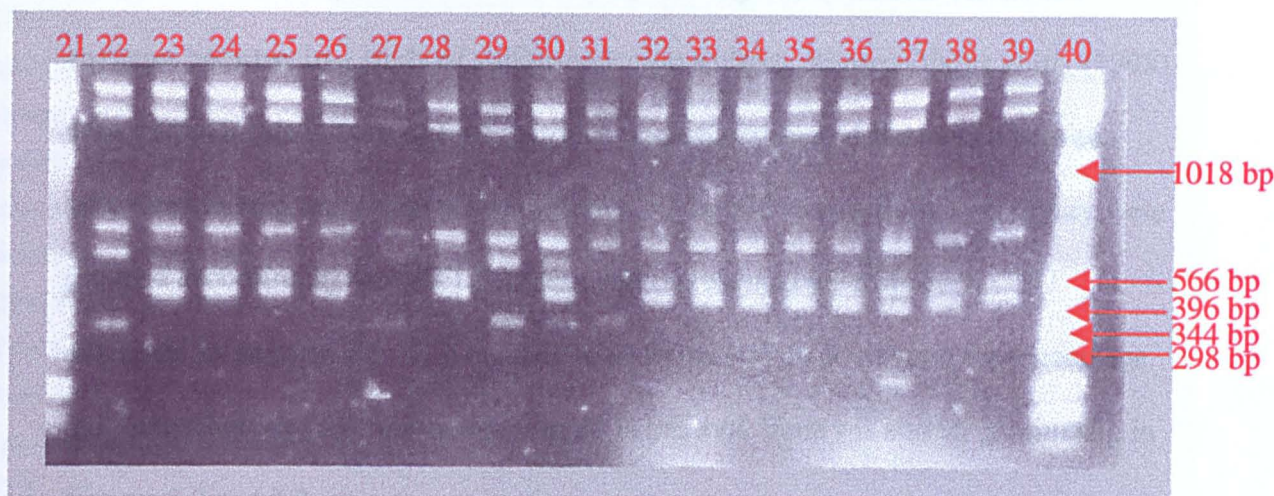


Figure 3.5. Restriction digests of 16S rRNA clones from the bacterioneuston library cut with *EcoRI* and *RsaI*. Lanes 1, 20, 21 and 40 are 1kb DNA ladder (molecular mass marker). Lanes 8 and 11 show cut vector with no insert. The sizes of the vector bands are 1,728, 1,440, 680 and 50 bp.

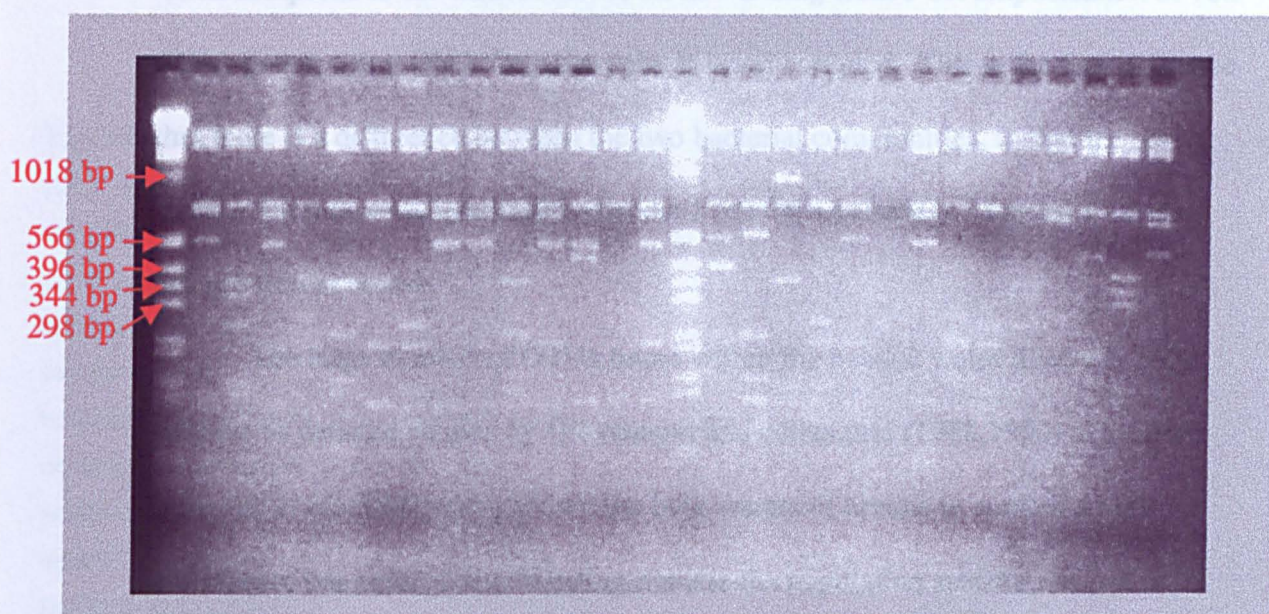


Figure 3.6. Examples of 16S rRNA clones from the pelagic library cut with restriction enzymes *EcoRI* and *RsaI* showing the RFLP for these clones.

The random clones chosen from the 16S rRNA clone libraries were cut with *EcoRI* to release the insert and digested with *RsaI* to give a distinct RFLP. Clones were also digested with *EcoRI*, *HincII* and *PvuII* to further separate the clones into

OTUs. The 16S rRNA inserts of the clones were digested with four restriction enzymes because, in a study of sulfate-reducing isolates, Rooney-Varga *et al.*, (1998) demonstrated that the use of four enzymes was necessary to differentiate between sequences having more than 95% similarity. Over 500 clones from each library were grouped according to their RFLP pattern and representatives from each OTU examined by sequencing. The number of different RFLPs found and the percentage that each ribotype pattern was represented in the total library is shown graphically in Figures 3.7 and 3.8.

The difference in the number of RFLPs found in the bacterioneuston compared to the pelagic libraries is striking (Figures 3.7 and 3.8). So is the domination of the sample by two OTUs from the sea surface microlayer environment when compared to the distribution of RFLPs arising from PCR amplification of 16S rRNA genes, using DNA from the pelagic water sampled. This is the first evidence that there is a difference between the two bacterial communities, in that the sea surface microlayer assemblage is distinct from the assemblage sampled from the pelagic waters below.

The large number of OTUs found within the pelagic water library is not a surprise as detailed studies by Giovannoni and colleagues (1988,1990) as well as Fuhrman *et al.* (1993) have shown the bulk waters of oceans to contain a high diversity of bacteria. The interesting point is the population structure of the bacterioneuston, hinted at by these results. Two groups of organisms, *Vibrio* and *Pseudoalteromonas* species, accounting for over 60 % and 20% respectively of the total library, dominate the assemblage (Figure 3.8).

Figure 3.7.

RFLP groupings of the 16SrRNA clones constructed from DNA amplified from the pelagic water collected. Each segment of the pie represents the percentage of the total number of clones analysed represented by each OTU.

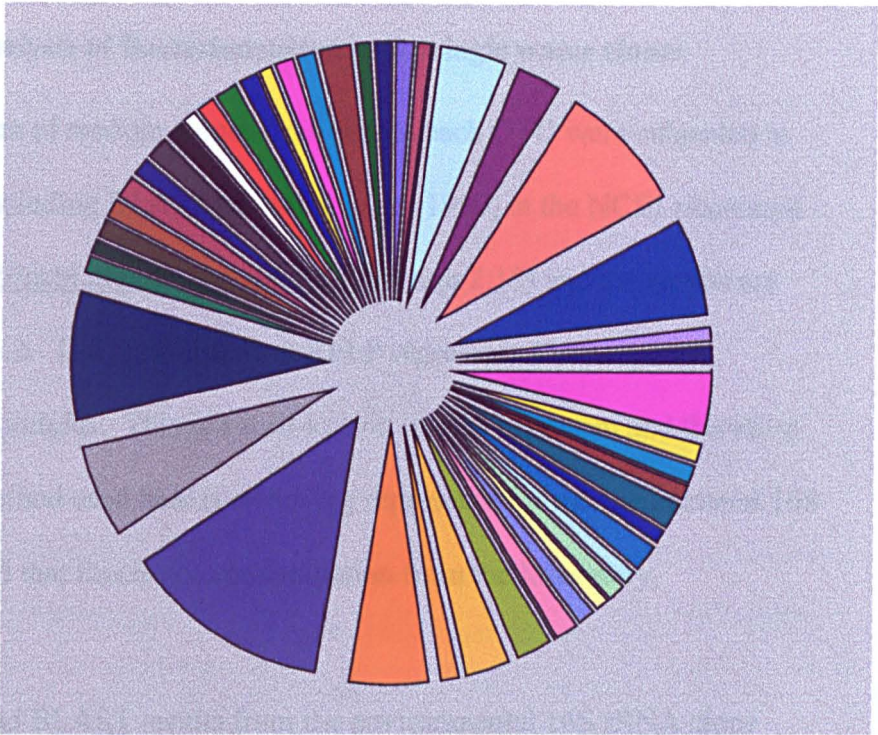
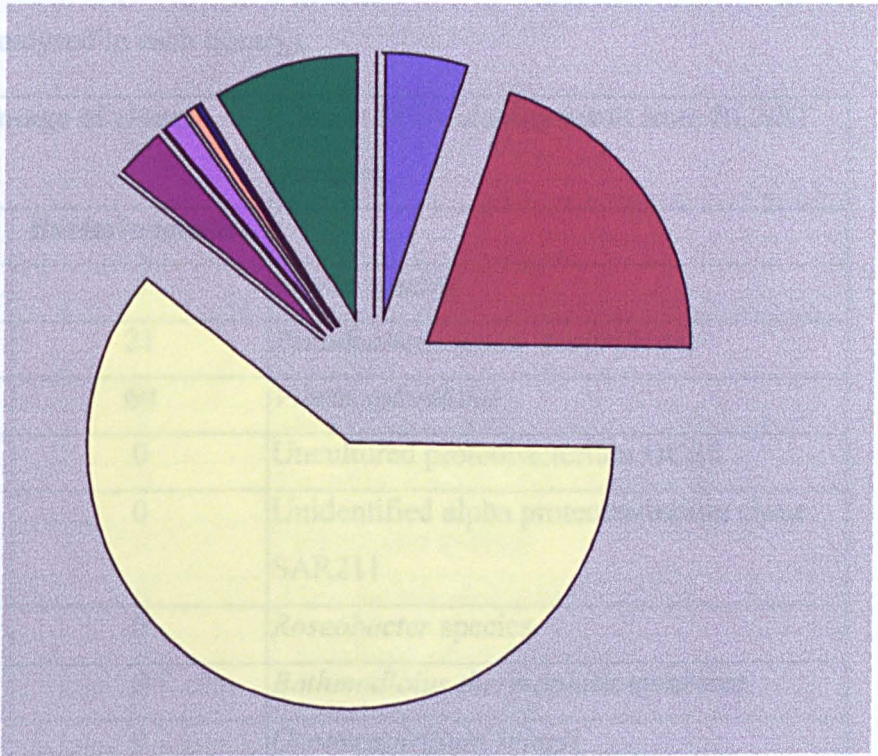


Figure 3.8.

RFLP groupings of the 16S rRNA clones constructed from DNA amplified from the bacterioneuston collected. Each segment of the pie represents the percentage of the total number of clones analysed represented by each OTU.



3.3.2. Sequence analysis of Bacterioneuston and pelagic water clones

Sequence data of random clones from within each OTU were subjected to database searches including BLAST (Altschul *et al.*, 1990) at the NCBI ribosomal RNA database (<http://ncbi.nlm.nih.gov/BLAST>, section 2.15) and the results are presented in Table 3.2. The most abundant RFLP represents *Vibrio* species, including *Vibrio splendidus*. This is a well-known marine organism and therefore indicates that the method used here is retrieving representative marine bacterial 16S rRNA sequences and that this is not contamination from the laboratory.

Table 3.2. RFLP and BLAST results from the environmental 16S rRNA clone libraries amplified from DNA extracted from the bacterioneuston and the pelagic water (500 clones analysed in each library).

O.T.U.	Percentage of clones		Closest DNA identity result from BLAST searches
	Pelagic	Bacterioneuston	
1	1	5	<i>Vibrio</i> species
2	1	21	<i>Pseudoalteromonas denitrificans</i>
3	1	60	<i>Vibrio splendidus</i>
4	4	0	Uncultured proteobacterium OCS5
5	3	0	Unidentified alpha proteobacterium clone SAR211
6	8	0	<i>Roseobacter</i> species
7	6	0	<i>Bathmodiolus thermophilis</i> symbiont
8	1	0	<i>Oceanospirillum kriegii</i>
9	1	0	Uncultured marine bacterium
10	4	0	<i>Rhodobacter</i> species
11	1	0	<i>Polaribacter</i> aff. <i>Irgensii</i>
12	1	0	Unidentified planctomycete
13	0	3	<i>Vibrio</i> species

14	1	0	<i>Oceanospirillum beijerinckii</i>
15	1.5	0	Unknown marine bacterium
16	1	0	Environmental clone
17	1.5	0	Uncultured proteobacterium OCS126
18	1	0	<i>Colwellia rossensis</i>
19	1	0	<i>Glaciecola fucicola</i>
20	1	0	<i>Mantoniella squamata</i>
21	1	0	Uncultured proteobacterium OCS28
22	1.5	0	Unknown alpha proteobacter
23	0	1.5	<i>Calyptogenia</i> species
24	0	0.5	<i>Stentrophomonas maltophilia</i>
25	0	0.5	<i>Sphingomonas paucimoblis</i>
26	0	0.5	<i>Vibrio anguillarum</i>
27	2	0	Uncultured marine bacterium HstpL4
28	3	0	<i>Mantoniella squamata</i>
29	1	0	<i>Polaribacter franzmannii</i>
30	5	0	<i>Roseobacter</i> species
31	13	0	Unidentified gamma proteobacteria HRV33
32	6	0	<i>Roseobacter shippagan</i>
33	7	0	Unknown bacterial species isolated from a soda lake
34	1	8	<i>Skeletonema pseudocostatum</i> chloroplast
35	1	0	Uncultured High G+C Gram positive
36	1	0	<i>Polaribacter</i> species
37	1	0	<i>Alteromonas macleodii</i>
38	1.5	0	<i>Cytophaga phaeoliformis</i>
39	1	0	<i>Sulfitobacter</i> species
40	1.5	0	Alpha proteobacterium MBIC3951
41	1	0	<i>Janthinobacterium lividum</i>
42	1	0	Unknown
43	1	0	Unidentified alpha proteobacterium
44	1.5	0	Uncultured gamma proteobacterium OM23
45	1	0	<i>Pseudoalteromonas</i> species

46	1	0	<i>Skeletonema pseudocostatum</i> chloroplast
47	1	0	<i>Vibrio anguillarum</i>
48	1	0	<i>Crassostrea virginica</i> symbiont
49	2	0	Unknown bacteria
50	1	0	<i>Oceanospirillum beijerinckii</i>
51	1	0	<i>Pseudomonas</i> species

3.3.3. Coverage; have enough samples been analysed?

By using the equations below, it can be estimated whether or not the number of clones investigated is statistically viable and whether or not the coverage of the samples is complete or if more samples are needed to complete the data set. The screening process of both environments was tested by statistical analysis to evaluate whether we had covered total diversity in the clone libraries by screening 500 clones. Species diversity can be considered to be composed of two components: species richness (the number of species in a community) and species evenness (the distribution of levels of abundance among the species). Two types of analyses can be used to assess diversity, rarefaction and coverage (Ravenschlag *et al.*, 1999). Rarefaction is a statistical technique for different applications in an ecological context and gives an estimation of the decrease in apparent species richness of a community with decreasing sub sample size. A second approach to evaluate whether diversity within a subsample approaches diversity within a sample of infinite size is to calculate coverage. Coverage (C) values are calculated by the equation:

$$C=1-(n/N) \times 100,$$

where n is the number of unique clones and N is the total number of clones examined.

$$C=1-(8/500) \times 100 = 98.4\% \text{ for the bacterioneuston libraries}$$

$$C=1-(46/500) \times 100 = 90.8\% \text{ for the pelagic water clone}$$

library

This second approach indicates we have over 90% coverage for both environments. Within the bacterioneuston or pelagic water assemblages, it is unlikely that analysing additional clone sequences will discover new major groups. Total phenotype richness, i.e., the number of phylotypes present, on the other hand, might reflect the potential within a microbial community to respond to changes in environmental conditions. At a different time point, those phylotypes not detected or represented by only one clone might play an important role in this habitat. Since coverage is based only on the number of unique clones relative to total richness, not taking evenness into account, it should be regarded only as a rough estimate within a sample of infinite size

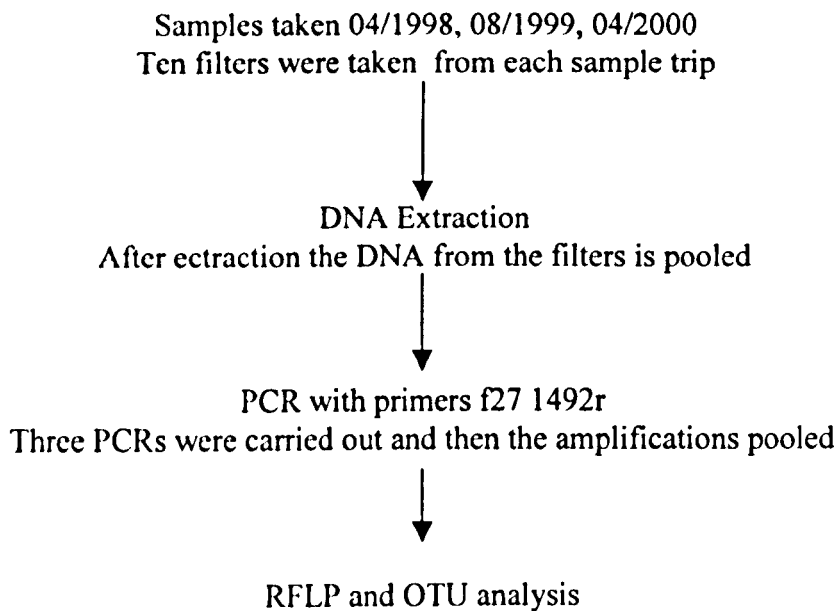


Figure 3.9. A summary of sampling and analysis of the sample sites

3.3.4. Comparative sequence analysis

For a detailed view of the clones isolated from the bacterioneuston and the bulk water phase and their phylogenetic relationships with the examples taken from databases and literature, a linear dendrogram including the clones identified from the environmental samples is presented in Figure 3.10 a-f (section 2.16).

The following pages contain a dendrogram depicting the relationship of the 16S rRNA sequences, from the majority of the clones found within this study with known 16S rRNA data from databases. This dendrogram gives an indication as to the type of organism found within this study from both the sea surface microlayer, that is the assemblage of the bacterioneuston and the pelagic waters below. Those clones labelled bacterioneuston are clones isolated from the bacterioneuston 16S rRNA clone library and those labelled pelagic were isolated from the pelagic water 16S rRNA clone library. The percentage of 100 bootstrap resamplings is indicated, however only values greater than 70% were used according to arguments presented by Zharkikh and Li (1992). This bootstrap analysis was used to provide confidence estimates for phylogenetic tree topologies. Evolutionary distances were calculated by the method of Jukes and Cantor (Jukes and Cantor, 1969), which corrects for the effects of superimposed mutations.

All sequences are over 500 bp in length, determined using the sequencing primer 357f (table 2.2).

Figure 3.10b

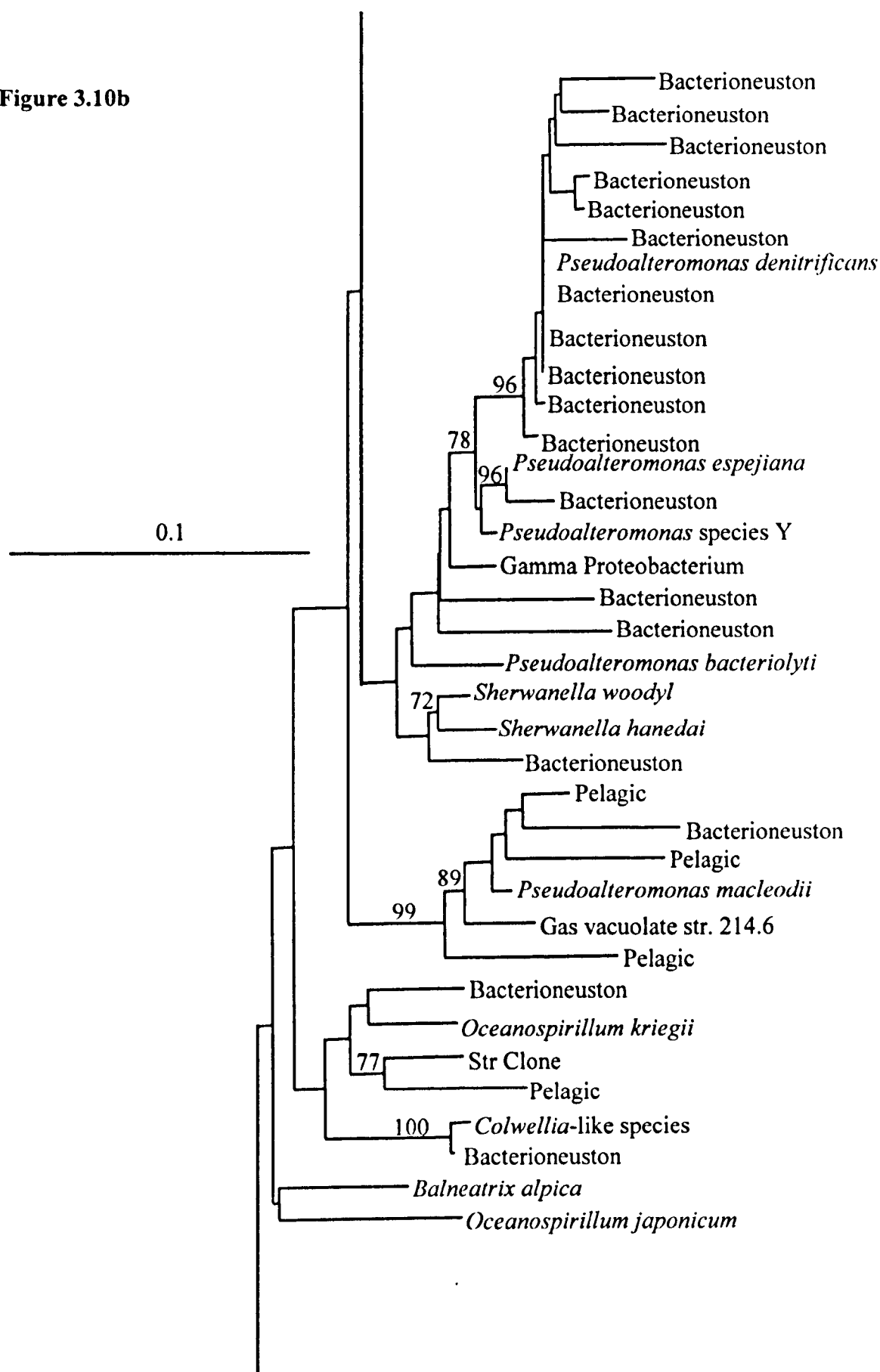
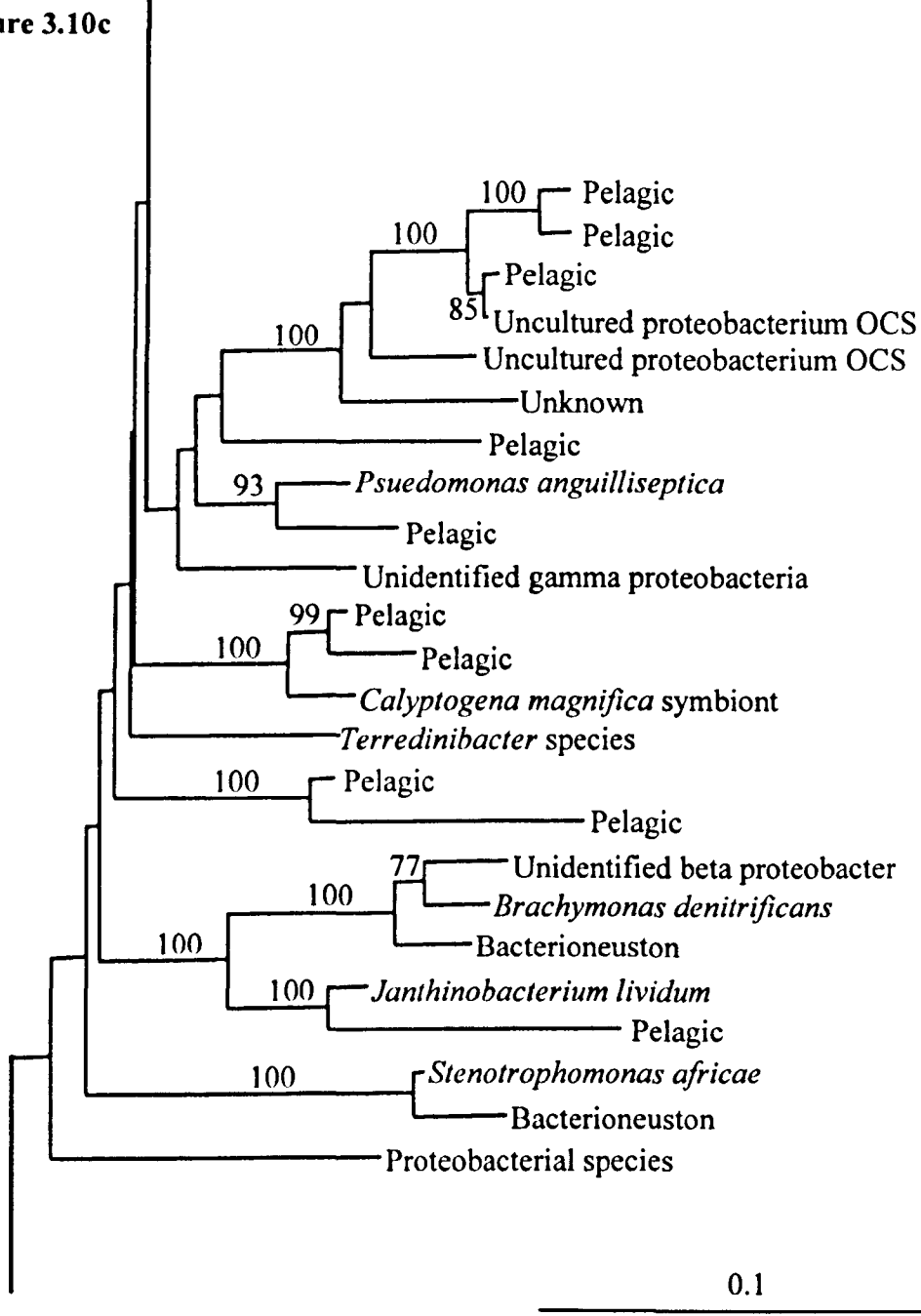


Figure 3.10c



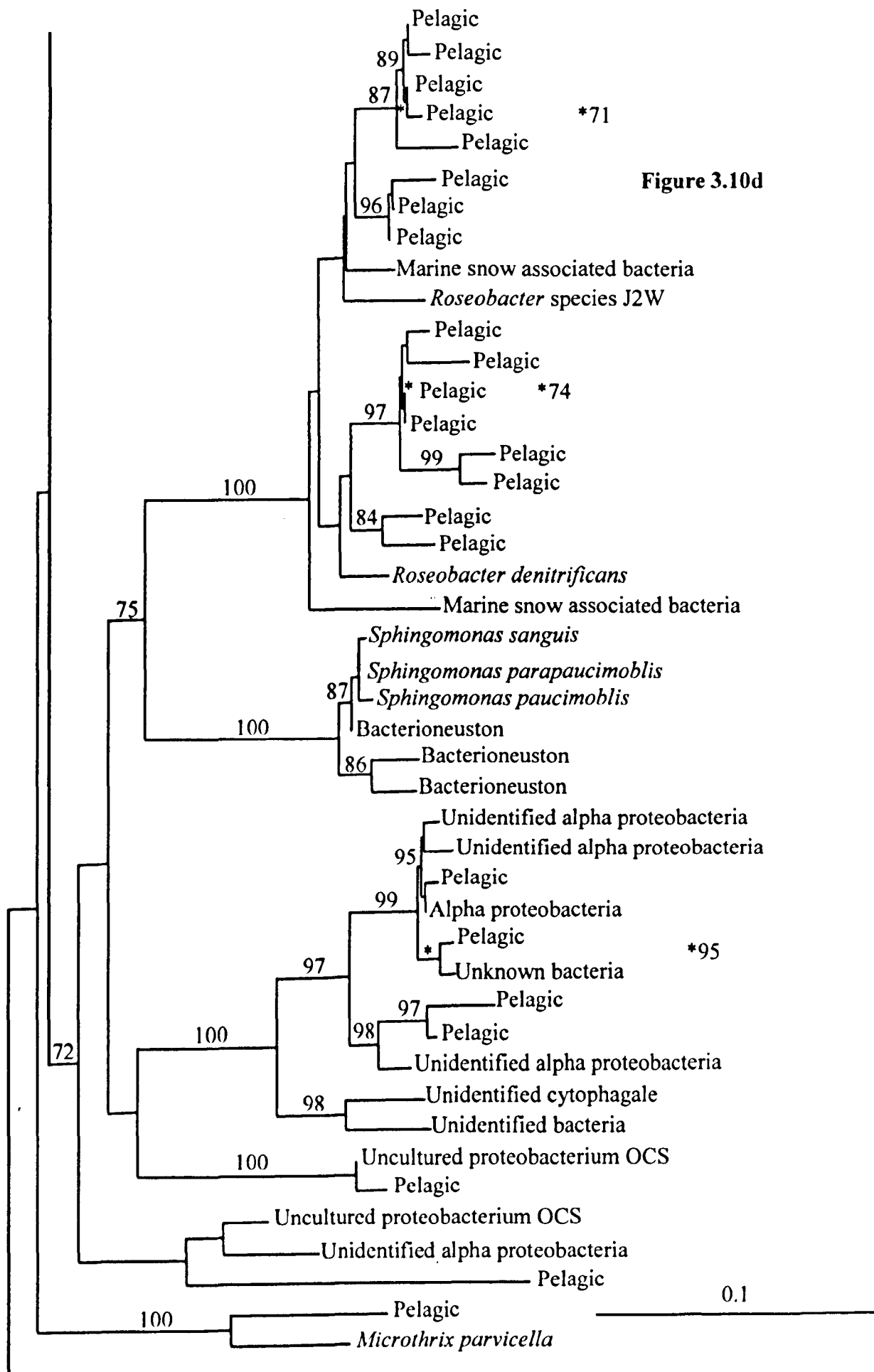
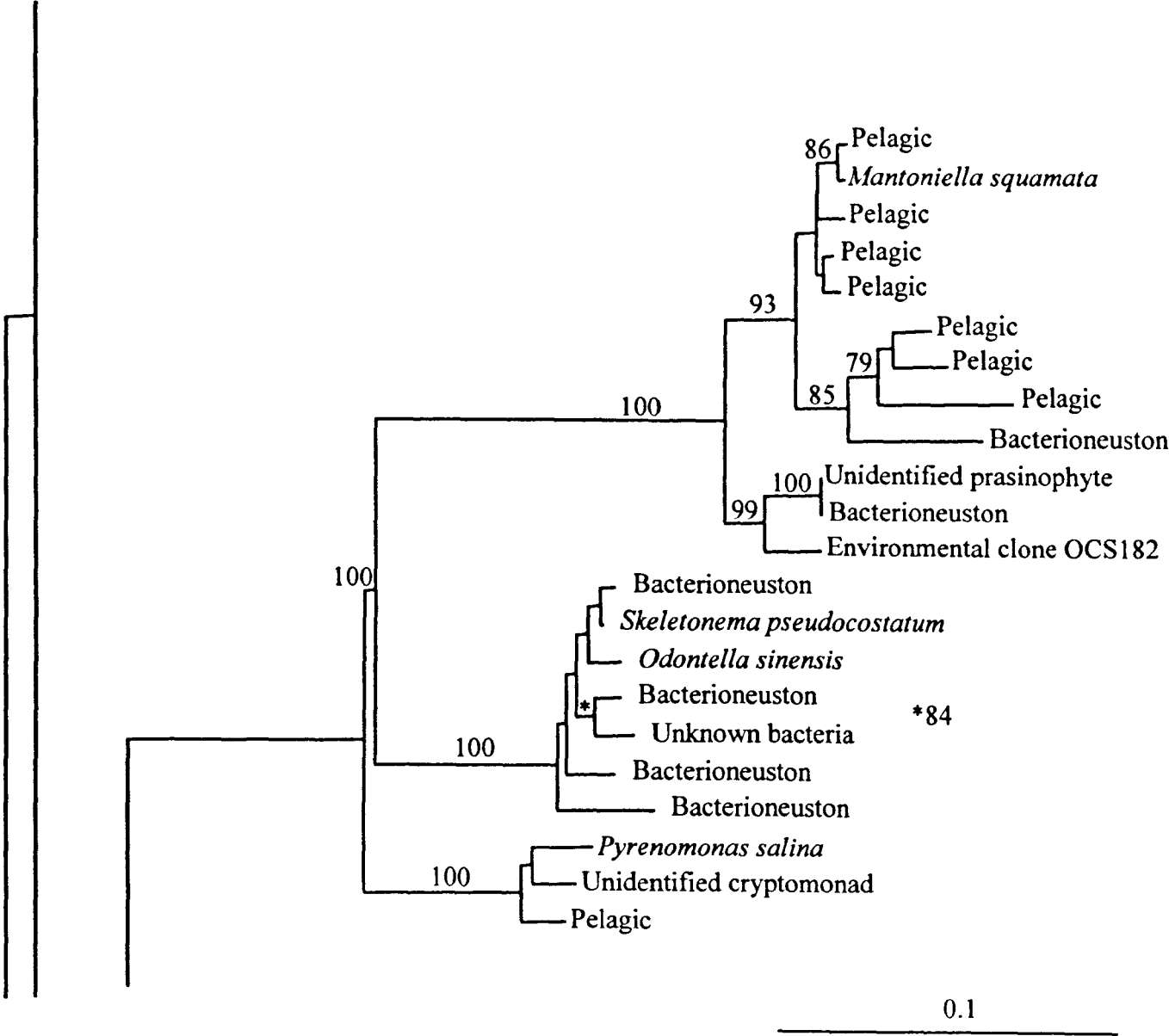
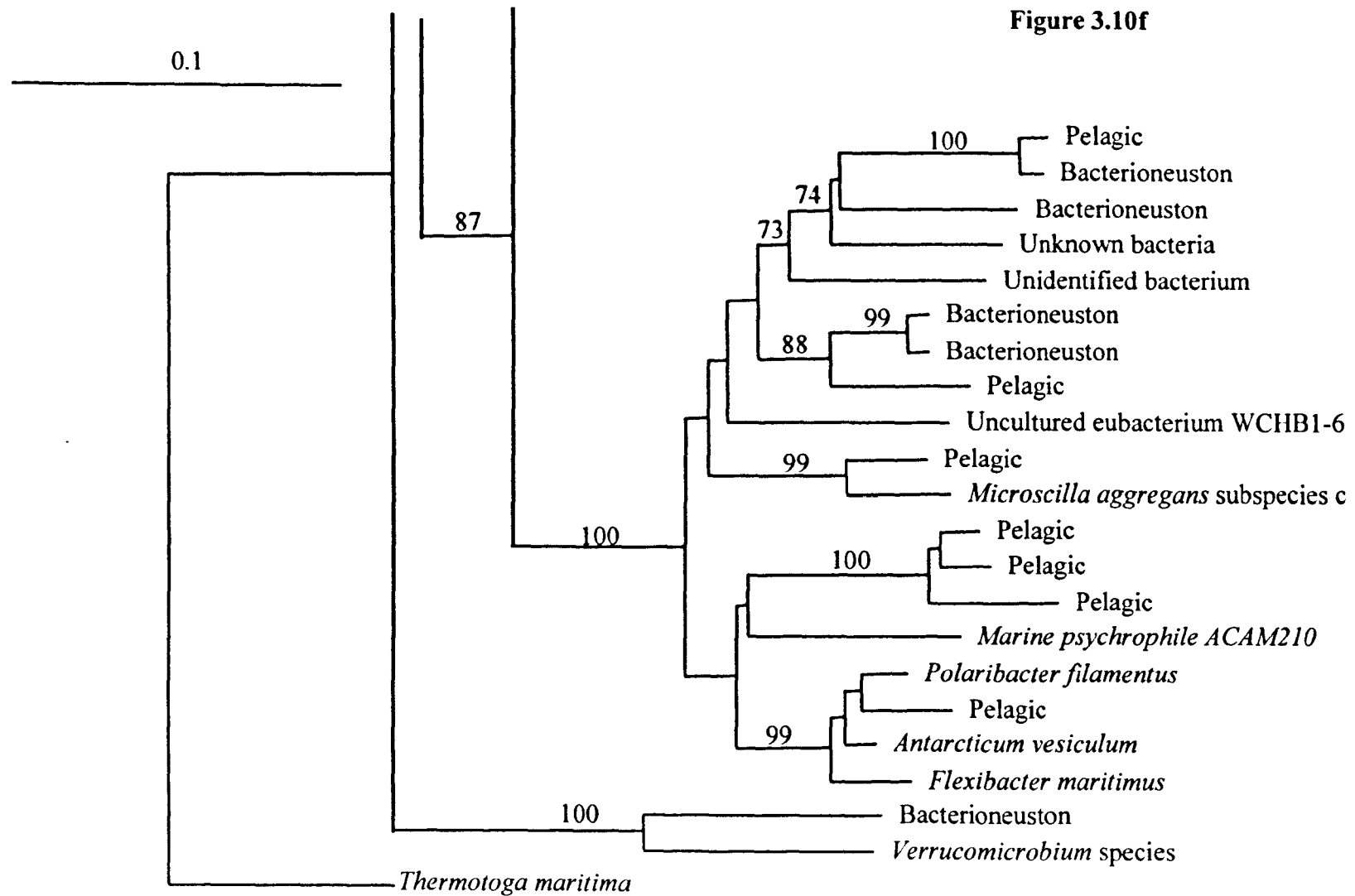


Figure 3.10e





3.3.5. Analysis of the 16S rRNA dendrogram

A number of sequences were removed from the 16S rRNA tree as they may have been chimeras and so were not included, as discussed in the materials and methods.

Sequencing of clones showed that bacterial populations belonged to various and diverse phylogenetic groups, including the α -, β - and γ -Proteobacteria, as well as the *Cytophaga-Flavobacterium-Bacteriodes* group and oxygenic phototrophic bacteria from the group comprising cyanobacteria and algal chloroplasts. The following sections describe some of the known organisms whose 16S rRNA sequences are similar to those 16S rRNA sequences found in the bacterioneuston or pelagic water. These reference organisms give an idea of the sort of organisms found within the bacterioneuston environment and in the pelagic waters. From these reference organisms it may be possible to infer some characteristics of those organisms found in the environment and if there is a particular trend.

As has been discussed in the Chapter 1 (section 1.5), to a bacterium, the air water interface, with its organic surface microlayer, may “appear” as a solid substratum. Thus, factors and characteristics that are known to be important in the adhesion of bacteria to solid substrata are also important in the adhesion of bacteria at the air water interface. Some of the factors that influence the adhesion of bacteria to the air water interface are:

- cell surface hydrophobicity and charge.
- cell surface structures.
- type and arrangement of film forming material.

The following sections give a brief description of some of the organisms from the major groups of sequences found in this study.

3.3.5.1. Brief description of the genus *Vibrio*

Vibrio splendidus is a halophilic *Vibrio* species that was first described as *Photobacter splendium* by Beijerinck in 1900. Strains of *Vibrio splendidus* have been isolated from seawater and fish on the east coast of North America and Denmark (Baumann and Baumann, 1981).

Most of the species of *Vibrio* are not closely related to each other in a phylogenetic sense (Baumann and Baumann, 1981; Baumann and Baumann, 1984; Brenner *et al.*, 1983). Thus, *Vibrio* is a heterogeneous genus, in a manner similar to the genus *Pseudomonas*. In the last two decades the vibrios and photobacteria have changed from a poorly characterised heterogenous group of organisms to several well understood natural groups. This has been due to the transfer of “non-fermentative vibrios”, “aerobic vibrios”, and “microaerophilic vibrios” to other genera such as *Campylobacter* (*Vibrio fetus*), *Wolinella* (*Vibrio succinogenes*), *Pseudomonas* and *Alteromonas* (Baumann and Baumann, 1981). The genera *Vibrio* and *Photobacterium* are classified in the family Vibrionaceae along with two other genera, *Aeromonas* and *Plesiomonas*. *Vibrio* is closely related to *Photobacterium*, which is expected due to their phenotypic similarities, but these genera are more distantly related to *Aeromonas* and to the family Enterobacteriaceae (Fox *et al.*, 1980). Most of the *Vibrio* species do not have close relatives, but there are a few exceptions: *Vibrio fluvialis* with *Vibrio furnissii*, *Vibrio splendidus* biogroup 1 with *Vibrio splendidus* biogroup 2. Even within a well-defined species, there can be considerable divergence. The type species for *Vibrio* is *Vibrio cholerae* and so the genus definition must be built around it this, however, it is not closely related to other *Vibrio* spp. (Farmer *et al.*, 1988).

3.3.5.2. *Pseudoalteromonas*

Bacteria readily isolated from marine waters are heterotrophic Gram-negative, flagellated bacteria (Bauman *et al.*, 1972) and can be divided into two subgroups depending on their capacity to ferment carbohydrates. Within the non-fermentative group, the genus designated *Alteromonas* has been revised, based on phylogenetic comparisons performed by Gauthier *et al.* in 1995. Their revision suggested that the genus *Alteromonas* should be divided into two genera, *Alteromonas* and a new genus, *Pseudoalteromonas*. This newly created genus has attracted significant interest for two reasons. First, *Pseudoalteromonas* species are frequently found in association with eukaryotic hosts in the marine environment and studies of such associations will elucidate the mechanisms important in microbe host interactions. Second, many species produce biologically active metabolites which target a range of organisms, for example, *Pseudoalteromonas denitrificans* produces autotoxic substances (Enger *et al.*, 1987).

The genus *Pseudoalteromonas* contains species that exclusively derive from marine waters and members have been isolated from marine locations around the world (Enger *et al.*, 1987). Species have been isolated from various animals such as mussels, pufferfish, tunicates and sponges, as well as from a range of marine plants (Holmstrom and Kjelleberg, 1999). Their existence in a variety of habitats and their worldwide spread suggest that the adaptive and survival expressed by *Pseudoalteromonas* species are diverse and effective.

Pseudoalteromonas denitrificans produces autotoxic substances, which kill the bacterial cells and inhibit further growth in dense culture (Enger *et al.*, 1987). Production of toxic compounds may allow for the bacteria to control large scale

processes, in contrast to the more restricted modification of their microhabitats caused by specific non-toxic extracellular agents.

3.3.5.3. *Listonella*

The genus *Listonella* was established round its type species *Listonella anguillarum* (*Vibrio anguillarum*), and included *Listonella damsela* (*Vibrio damsela*) and *Listonella pelagia* (*Vibrio pelagius*). It was defined almost entirely on the basis of rRNA structure. Interestingly *Listonella* does not include *Vibrio ordalii*, which is highly related to *Vibrio anguillarum* by DNA hybridisation, and is the only close relative based on this technique.

Listonella anguillarum is a marine vibrio that causes disease in marine fish and other marine animals (Balows, 1981). The most important is a septicemic disease in marine fish characterised by a deep necrotising myostitis and subdermal haemorrhages. This disease is particularly important when it occurs at fish farms, as the economic losses are worldwide and considerable. The main predisposing factor seems to be a rise in water temperature to 15 °C or more (Sakazaki and Balows, 1981).

3.3.5.4. *Colwellia*

The genus *Colwellia* contains obligately barophilic bacteria (Deming *et al.*, 1988). The taxonomic position of this genus is not yet clear; it varies with the clustering method applied to the 5S rRNA base sequences: it is either a member of the Aeromonadaceae (Colwell *et al.*, 1986) or equidistantly removed from the Aeromonadaceae and the Enterobacteriaceae (MacDonell and Colwell, 1985), or

outside the three large families Aeromonadaceae, Vibrionaceae and Enterobacteriaceae (Deming *et al.*, 1988).

3.3.5.5. *Oceanospirillum*

Oceanospirillum (Hylemon *et al.*, 1973) is a genus of which some of its members have a long history as former species of the genus *Spirillum*. The organism is isolated from coastal seawater, from decaying seaweed, and from putrid infusions of marine mussels (Hylemon *et al.*, 1973). During the last 25 years, the classification of both freshwater and marine spirilla has changed considerably, in the original description (Hylemon *et al.*, 1973) *Oceanospirillum* contained five species, another six species have been added and are described by Krieg in Bergey's Manual of Systematic Bacteriology. However, Pot *et al.*, (1989), showed that four of the species including *Oceanospirillum kriegii* were erroneously included in the genus *Oceanospirillum*.

3.3.5.6. *Janthinobacterium*

Janthinobacterium lividum strains have been isolated from water and soil from rivers, creeks, lakes, and springs as well as the marine environment (Gonzalez *et al.*, 1987).

3.3.5.7. *Roseobacter*

The genus *Roseobacter* is comprised of aerobic, marine, pink-pigmented bacteria which contain bacteriochlorophyll a (Shiba, 1991). At present the genus consists of two species, *Roseobacter denitrificans* and *Roseobacter litoralis*.

Roseobacter denitrificans has previously been called *Erythrobacter* sp. Ochl14 (Shiba and Simidu, 1982), because like *Erythrobacter*, *Roseobacter denitrificans* is aerobic and contains bacteriochlorophyll a. This genus has also been named “the marine alpha bacteria” (Gonzalez and Moran, 1997). This phylogenetic cluster of organisms, belonging to the α -subclass of the class Proteobacteria, is abundant in coastal seawater in the southern USA, where it can account for up to 30% of the 16S rDNA (Gonzalez and Moran, 1997). This group is also well represented in coastal and open ocean 16S rDNA clone libraries obtained from a number of marine ecosystems (Mullins *et al.*, 1995; Suzuki *et al.*, 1997).

3.3.5.8. *Verrucomicrobium*

The genus *Verrucomicrobium* contains heterotrophic bacteria with multiple appendages (prostheco bacteria). They have been isolated from marine environments (Albrecht *et al.*, 1987).

3.3.5.9. *Microthrix*

Acidimicrobium thermooxidans is remotely related to *Microthrix parvicella* (87% 16S rDNA sequence similarity; Blackall *et al.*, 1994). *Microthrix parvicella* is a Gram-positive straight filamentous bacterium that is the major causative agent of foaming and bulking in activated-sludge sewage-treatment plants (Christensson *et al.*, 1998).

3.3.5.10. *Serratia*

Serratia grimesii is a member of the family Enterobacteriaceae and associated with marine water. The genus *Serratia*, is comprised of a group of bacteria that are related both phenotypically and by DNA sequence. The type species of the genus is *Serratia marcescens* (Williams and Warner, 1980).

3.3.5.11. *Pseudomonas*.

The Pseudomonadaceae are a very large and important family of Gram-negative bacteria. Members of the family comprise a substantial proportion of the microflora of free-living saprophytes in soils, fresh water, marine environments, and many other natural habitats, and they may also be found in association with plants or animals as agents of disease.

The basic morphological characteristics common to all Pseudomonads are the rod shape of the cells, which can be either straight or slightly curved in one plane, the presence of polar flagella, a Gram-negative staining reaction, and the absence of spores.

3.3.5.12. *Teredinibacter*

Teredinibacter turnerae cells are Gram-negative curved rods that are motile by virtue of a single polar unsheathed flagellum. Analysis of 16S rRNA sequences places *Teredinibacter turnerae* in the gamma subdivision of the proteo bacteria. *Oceanospirillum linum* is among the species most closely related to *Teredinibacter turnerae* although this relationship is relatively distant (Distel, 1990).

3.3.5.13. *Thermotoga maritima*

Members of the *Thermotoga* group are the most extremely thermophilic eubacteria presently known, with growth up to 90°C and an optimal growth temperature around 80°C. *Thermotoga maritima* was originally isolated from a geothermally heated, marine sediment at Vulcano, Italy (Huber *et al.*, 1986). Of the eubacteria sequenced to date, *Thermotoga maritima* has the highest percentage (24%) of genes that are most similar to archeal genes. Conservation of the gene order between *Thermotoga maritima* and archaea in several clustered regions of the genome suggests lateral gene transfer may have occurred between thermophilic eubacteria and archaea. Also no DNA sequences were found within this study which showed homology to *Thermotoga maritima* and the whole of the 16S rRNA gene is available from the database. For these reasons, *Thermotoga maritima* was chosen as the out-group.

In Chapter 1 (section 1.5) a review of work previously carried out on the bacterioneuston was presented. Part of this work indicated the presence of extracellular polymers, fibres and fimbriae associated with the bacterioneuston (Fuerst *et al.*, 1987). From the results presented here, many of the genera found within the bacterioneuston also produce extracellular polymers, fibres or fimbriae. Within this review, the bacterial composition of the bacterioneuston was discussed and previous literature found *Pseudomonas*, *Vibrio*, *Aeromonas*, *Achromobacter*, *Flavobacterium*, *Bacillus* and *Spirillum* to be dominant. These genera were found in this study, but within our definition of the bacterioneuston and our sample area the genera *Vibrio* and *Pseudoalteromonas* dominated the assemblage at the sea surface. All those

genera found within the pelagic waters 16S rRNA clone libraries have been found within previous investigations into the bacterial composition of the oceans.

From the dendrogram describing the 16S rRNA sequences found (Figures 3.9 and 3.10a-f) we have demonstrated that the bacterioneuston is a distinct population from the bacterial population of the pelagic waters below. Within this distinct community we can explore the composition of the bacterioneuston as an environment distinct from the bulk water.

3.3.6. DGGE analysis of the bacterioneuston versus the pelagic water

To distinguish whether or not the bacterioneuston community is different from that of the bulk waters below, we used the technique of Denaturing Gradient Gel Electrophoresis (DGGE, sections 1.20.3, 2.17). This technique allows investigation into an environment in a different way to library screening and so can be used alongside other molecular techniques without overlapping, to complement the investigation. For the purpose of this study, DGGE was used solely to demonstrate the difference or similarity between the two communities, sea surface microlayer and pelagic water, of bacteria samples from the environment. By amplifying a fragment of the eubacterial 16S rRNA genes present in the DNA samples extracted from the bacterioneuston or the pelagic water using PCR primers specifically designed for this (Table 2.2) the resultant gene fragments could be compared according to their G+C content. Without specific (known) markers, we cannot comment on the bands present, only on the comparison of the two libraries.

DGGE analysis of the 16S rRNA bands amplified from the two environments (Figure 3.11) shows a difference in the make up of both environments at the two

sampling times and also validates the sampling method from the point of view of membrane bias.

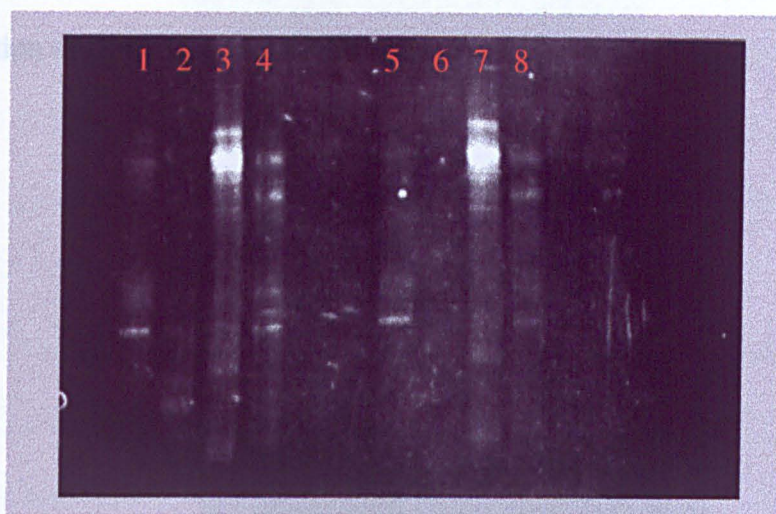


Figure 3.11. DGGE gel showing elucidation of PCR amplified 16S rRNA bands from the environment using the PCR DGGE primers GC-GM5f and 907r (Table 2.2). Lanes 1 and 5 were Bacterioneuston samples taken in 1999; lanes 2 and 6 were Bacterioneuston samples taken in 2000; 3 and 7 were pelagic samples taken in 1999; and lanes 4 and 8 were pelagic samples taken in 2000.

The variation within the one environment does not pose a problem to this study as the sample sites were as closely replicated, i.e. same co-ordinates, as possible, but obviously the water will have changed over the year. It is not like terrestrial sampling where you can re-examine the same plot of earth. Also with differing conditions being applied to the sea surface compared to the water column, the organisms, which respond to the different conditions in advantageous ways, will dominate. The sequence analysis of the second bacterioneuston sample showed a high proportion of sequences which had a large amount of similarity to those published for *Skeletonema pseudocostatum*, this may suggest the start, or at least give some evidence, of an algal bloom.

Figures 3.12 and 3.13 demonstrate the obvious difference between the two environments detected by DGGE analysis. The 16S rRNA PCR amplified pelagic DNA sample produced more bands of higher intensity than those produce by the amplification of DNA from the bacterioneuston.

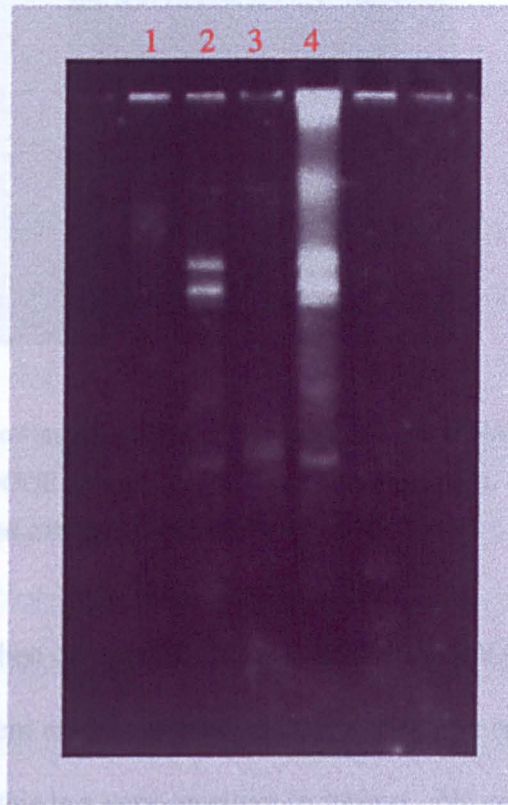


Figure 3.12. DGGE gel showing elucidation of PCR amplified 16S rRNA bands from the environment using the PCR DGGE primers GC-GM5f and 907r (Table 2.2). Lanes 1 and 3 were Bacterioneuston samples and 2 and 4 were Pelagic samples. The DNA in lanes 2 and 4 has been concentrated approximately ten fold.

These two figures (3.12 and 3.13) demonstrate the sample variation between two gels.

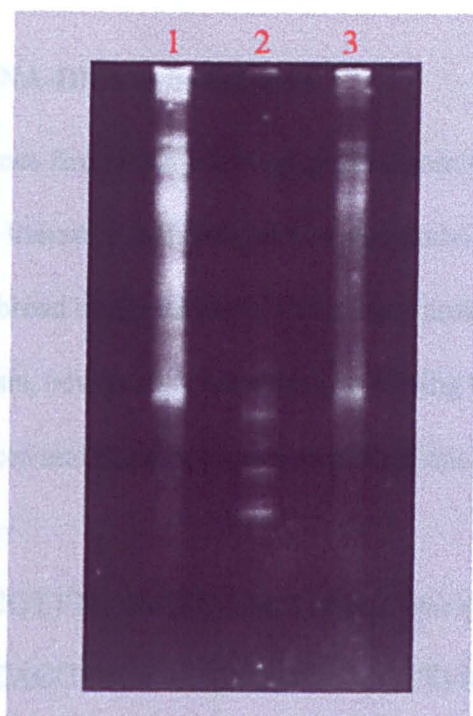


Figure 3.13. DGGE gel showing elucidation of PCR amplified 16S rRNA bands from the environment using the PCR DGGE primers GC-GM5f and 907r (Table 2.2). Lanes 1 and 3 were pelagic samples, and lane 2 was a bacterioneuston sample.

As can be seen when comparing all the DGGE 16S rRNA PCR amplified bands between the different environments and within the same environment, but different amplifications, this is a very sensitive technique. No conclusions about the community structure can be drawn from the gels, as there are no standards to compare bands with. However, we can use this technique to compare environments to show difference in community make up and to show change in community over time. Bands were excised but attempts to sequence these bands did not result in sequences of sufficient quality to draw conclusions from them. This is most probably due to having to use silver staining to resolve the bands as the resolution was very faint using EtBr or Syber-gold/green, and the difficulty of resequencing bands which have been stained using silver is well known.

3.3.7. Colony blot DNA-DNA hybridisation

Over 1000 clones from each site were picked onto filters and probed using probes reported in the literature and designed in this study. The filters were probed to give an idea of the broad composition of the sample assemblages. On the basis of initial sequence analysis, new probes were designed using the ARB database, using known isolate sequences and sequences obtained from this study. The two probes designed were:

Vibsp1 (TGTCGTTTCCGCTCGACT) for *Vibrio splendidus* and other *Vibrio* species; and Psal1 (CCAGCTTCTAGTAGACATCGTT) for most *Pseudoalteromonas* species.

Clones were picked from the colony blots to confirm the identity of the 16S rRNA, via sequencing, and the validity of the probes.

Probing of the Bacterioneuston 16S rRNA clone library with probe Vibsp1 resulted in hybridisation detecting 57% of the clones. Probe Psal1 hybridised with 32% of the Bacterioneuston 16S rRNA clone library. Of the screened pelagic extracted DNA clones, 13% hybridised to the Vibsp1 probe and 8% to the probe Psal1. These very preliminary results support the data collected by RFLP analysis that indicated that a large percentage of clones isolated from the bacterioneuston were related to *Vibrio* species sequences, with the rest of the assemblage dominated by sequences related to *Pseudoalteromonas* species. Whereas in the analysis of the pelagic 16S rRNA clone libraries neither probe hybridised to a large percentage of the library, as expected from the earlier clone analysis.

3.4. Discussion

A major question in microbial ecology is to identify which members contribute to the activity and/or production of the whole bacterial community. During the last decade, the application of cultivation-independent molecular techniques using 16S rRNA or its encoding gene as molecular markers have provided new insights into microbial diversity and in the structure and dynamics of microbial communities.

In an open system there is no physical boundary between communities. In order to test the hypothesis that the bacterioneuston constitutes a distinct community the question must be framed in the context of an environmental gradient, which operationally defines the boundary. Then samples of the assemblage on either side of the boundary can be tested to see if they support the hypothesis of distinct communities.

The community is a group of interacting organisms (net interaction within community greater than external interactions). It is defined by physical space and biology. Whereas, the assemblage describes no implication of special interaction. The assemblage is simply what organisms are in the space. It is defined by physical space only.

The neuston refers to the biota at the surface. The term Bacterioneuston assemblage is non-controversial as it simply means whatever bacteria are at the surface. Whereas the neuston community implies a specially adapted population which has significant internal processes that are distinct from the water column.

The initial 500 clones from the libraries showed a skewed distribution with the sample from the bacterioneuston having a smaller assemblage than that of the pelagic water sampled. The results from the RFLP analysis, the probing experiment

and the DGGE all show the bacterioneuston to be a distinct population varying from the bacterial population of the bulk water phase below.

To see if the bacteria are attracted or repelled by the membrane the effect of the membrane on sampling was tested on a freshwater pond as well as at sea. In the pond a membrane was placed on top of the water, subsurface water was collected and filtered through a membrane and a membrane was submerged into sub surface water collected. The two membranes in contact with the subsurface waters showed very similar RFLP results and the membrane placed on the surface showed less variation in the RFLP patterns resolved. Two of the samples collected from the bacterioneuston on different days showed a different banding pattern on DGGE gels and showed a slightly different bias in RFLP and sequence analysis. The DNA extraction was carried out in the same way for all the samples taken.

One theory for the distinct assemblage of the bacterioneuston when compared to the pelagic water could be the formation of a biofilm at the sea surface interface. Biofilms are usually thought of as the slimy layer of microorganisms that cover solid surfaces, in this case the surface tension forces may act like a solid surface (Hardy, 1982). However, there are a number of features that distinguish biofilm populations from their planktonic (suspended or free floating) counterparts, namely: the association with a surface, high population densities, an extracellular polymer (EPS) slime matrix, and a wide range of physical, metabolic and chemical heterogeneities (Hardy, 1982), although, some biofilms may not have all these features. Indeed a concise universal definition of biofilms has yet to emerge. In part this is because of the wide diversity of biofilm populations. Although much of contemporary microbiology is based on the study of planktonic "cells", it is now thought that biofilms are the primary habitat for many microorganisms. Microbial mats

associated with sediment and suspended microbial flocs or aggregates, although different in appearance from conventional biofilms, have many important features in common and thus are included in the definition of "biofilm." Often biofilm cells are embedded within a highly hydrated EPS matrix, and in the absence of corrosion products or scale, biofilms are estimated to be primarily water. The physical properties of the biofilm are largely determined by the EPS, while the bacterial cells determine the physiological properties. Many *Pseudoalteromonas* species have been demonstrated to produce antibacterial products, which appear to aid them in the colonisation of surfaces including those of their hosts. The production of agarases, toxins, bacteriolytic substances and other enzymes by many *Pseudoalteromonas* species may assist the bacterial cells in their competition for nutrients and space as well as in their protection against predators grazing at surfaces (Holmstrom and Kjelleberg, 1999).

The bacterial ecology at surfaces is complex and many additional factors besides the production of secondary metabolites and other secreted products affect bacterial responses. For example, Ivanova *et al.* (1998) demonstrated that the degree of hydrophobicity of the substratum influences the production of anti-bacterial metabolites. The highest anti-microbial activity was found to occur on hydrophilic surfaces despite the fact that attached *Pseudoalteromonas* cells were more abundant on hydrophobic surfaces. This finding may suggest that the expression of the anti-microbial activity may be switched on and off depending on fluctuations and stimuli present in the immediate environment of the cell (Holmstrom and Kjelleberg, 1999).

The range of biological activities carried out by *Pseudoalteromonas* species suggest that the expression of bacterial extracellular compounds allows for the producer to successfully compete with other organisms. However, bacteria can also

produce compounds which aid in the survival of other marine organisms. For example, the production of exopolysaccharides has been demonstrated to enhance the chances for other organisms to survive in specific marine habitats (Holmstrom and Kjelleberg, 1999).

Jannasch and Jones (1959) first pointed out the discrepancy between direct microscopic enumeration and plate counts of bacteria, isolated from the marine environment. They attributed it to the presence of bacteria in aggregates, to selective effects of the media used, and to the presence of inactive cells. In 1982, Colwell and co-workers developed the viable but non-culturable hypothesis (Xu *et al.*, 1982). Ferguson *et al.*, showed that >99.9% of the natural bacterioplankton community in seawater could not be cultured on Marine Agar 2219 (1984). The culture-independent methods, most notably 16S rRNA gene clone libraries, revealed the high diversity of marine bacterioplanktonic communities (Britschgi and Giovannoni, 1991; Fuhrman *et al.*, 1992; Fuhrman *et al.*, 1993; Giovannoni *et al.*, 1990; DeLong, 1992). In the Atlantic and Pacific Oceans, most of the sequences clustered within the alpha and gamma subclasses of Proteobacteria and two novel groups of Archea were found. 16S rDNA sequences of previously isolated marine bacteria (e.g., *Pseudomonas*, *Rhodobacter*, *Vibrio* and *Arthrobacter* spp.) were occasionally retrieved, but their *in situ* abundances remained unknown (Bowman *et al.*, 1997; Suzuki *et al.*, 1997).

It has been indicated throughout this chapter that the surface film microenvironment is quite distinct from the bulk phase. Specific components of the interface environments and their possible effect on microorganisms have been reviewed. On the one hand, the high levels of nutrients, both organic and inorganic, compared to the bulk phase would contribute to an increase in the metabolic activity

of the bacterioneuston. On the other hand, there are many detrimental factors associated with life at the air-sea interface: unstable salinity and temperature conditions, enrichment of both toxic organic substances and heavy metals, and intense UV and visible radiation.

Chapter Four

Gas Exchange

4.1. Laboratory gas exchange tank

This chapter describes the laboratory gas exchange tank rationale, the methodology, and the preliminary biological experiments. It will also contain the initial gas exchange experimentation from testing the theory on the open ocean.

All experiments were carried out in conjunction with Dr Tom Frost (University of Newcastle upon Tyne).

4.1.1. The tank and detection systems

The use of the laboratory tank and analytical system for invasive and evasive gas exchange experiments is explained here briefly (for more a more in depth explanation see Frost, 2000).

4.1.2. Theory

A gas exchange experiment within a tank is initiated by modifying the partial pressures (pp) of the elected gases in either the water or air phases, thereby creating disequilibrium across the air-water interface. This disequilibrium causes the transfer of gas across the interface in response to the concentration gradient via diffusion. By measuring the pp in both phases at intervals throughout the experiment, it is possible to derive a gas flux. We can, therefore, calculate the total gas transfer velocity relative to the water phase (k_w) or the air phase (k_a).

4.1.3. Previous work

Conventional gas exchange tank experiments have focused on studying k_w in “open” systems (usually a large water tank open to the atmosphere), where changing the pp of the selected tracer gas is restricted to the water phase (Holmen and Liss,

1984). It is fairly straightforward to add gas to a confined water phase, compared to controlling an open air phase, so most “open” tank experiments have measured evasive (water to air) gas fluxes (Ho *et al.*, 1997). Although invasive (air to water) flux measurements are possible by de-gassing the water phase, this is only applicable to those gases which are in sufficient concentration in the atmosphere to produce a measurable increase in the water phase.

An “open” tank system can also take the form of a floating chamber, annexing a volume of atmosphere next to a body of water (e.g. the ocean) (Conrad and Seiler, 1988). In such experiments, the atmosphere in the chamber can be modified to measure both invasive and evasive k_w , providing the gas of interest is in sufficient concentration in the surface water as no control can be exerted over the water phase composition.

The experiments presented in this chapter, however, require the measurement of both invasive and evasive gas fluxes for specified gases for both k_w and k_a , and require the total amount of gas within the whole system to be monitored. To accomplish this a “closed” tank system was developed and used which contained both a hydrosphere and atmosphere. Within such a system, invasive and evasive experiments could be conducted by the modification of either the water or the air phase with specific trace gases. The closed nature of this system also allowed us to monitor the total amount of certain gases in the system.

4.1.4. Overview of the laboratory system

The closed laboratory gas exchange system used is shown schematically in Figures 4.1 and 4.2. Essentially the system linked two Gas Chromatographs to the

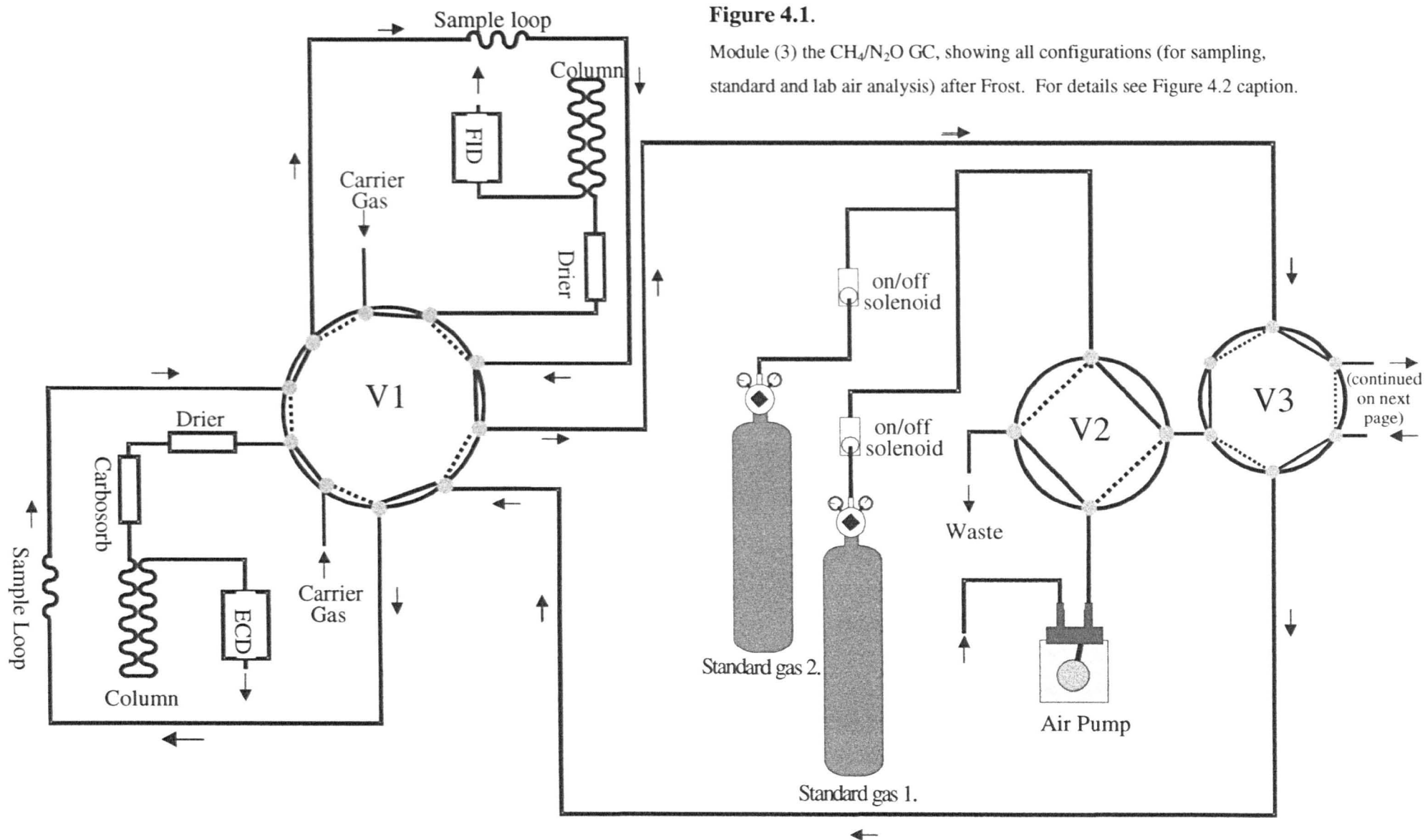
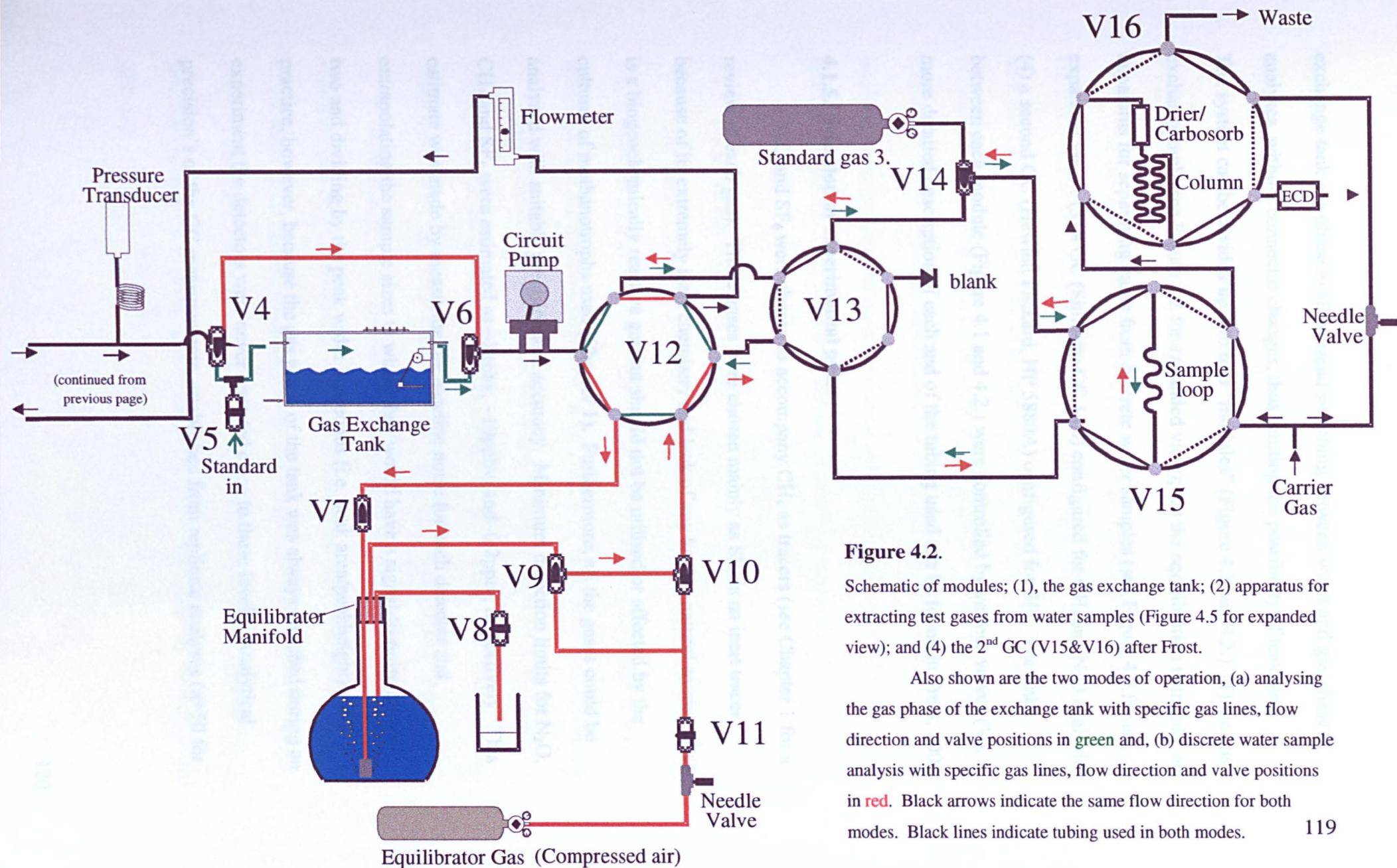


Figure 4.1.

Module (3) the $\text{CH}_4/\text{N}_2\text{O}$ GC, showing all configurations (for sampling, standard and lab air analysis) after Frost. For details see Figure 4.2 caption.



exchange tank gas phase to allow rapid switching between water and gas phase analyses, without connector changes, thus limiting the possibility of leakage.

The system can be divided up into four “modules” (Figure 4.1 and 4.2.), (1) the gas exchange tank (see Figure 4.3 for expanded view); (2) the equilibration manifold, an apparatus for separating gases from discrete water samples (see Figure 4.5 for an expanded view); (3) a GC (Shimazu GC 14B) configured for CH₄ and N₂O analysis; (4) a second GC (Hewlett Packard, HP 5880A) configured for SF₆. The links between each module (Figure 4.1 and 4.2.) were controlled by various valves (V), a more detailed description of each and of the tubing used can be found in Frost, 2000.

4.1.5. Selection of experimental gases

N₂O and SF₆ were chosen to accompany CH₄ as tracers (see Chapter 1 for a review of each gas). These gases were chosen mainly as SF₆ is an inert tracer, because of its extremely inert chemistry and lack of any known natural source, N₂O is a biogeochemically reactive gas but should not be utilised or affected by the cultures of methanotrophs used (Chapter 1). Furthermore, all the gases could be analysed with suitable precision and accuracy. Minimum detection limits for N₂O, CH₄ and SF₆ were estimated as ~2ppbv, ~10ppbv, and ~0.2pptv, respectively. This estimate was made by measuring the baseline noise for each detector and extrapolating the sample sizes to where they would have a signal-to-noise ratio of two and dividing by the peak width in seconds (i.e. peak area/peakheight). In practice, however, because the gas phase of the tank was always enriched during an experiment the detectors were never operated close to these levels. Analytical precision's of the GC measurements, established from replicate analyses (n=50 for

N₂O and CH₄, n=20 for SF₆) of standards were better than $\pm 0.6\%$ ($100\sigma/\text{mean}$) for N₂O, $\pm 0.8\%$ ($100\sigma/\text{mean}$) for CH₄ and $\pm 0.4\%$ for SF₆.

4.1.6. The tank

The laboratory tank (Figure 4.3.) had internal dimensions of 98cm (L) x 48 cm (H) x 48 cm (W). It comprised a sealed Perspex tank to which seawater could be added to create a variable ratio of air phase volume (V_a) to water volume (V_w), measured by tank dimensions (error $< \pm 40$ ml). The gas composition of either phase was varied by the controlled introduction of known volumes of standards of predetermined gas concentration. The water phase could also be varied by the addition of specific water types and, in theory, bacterial communities.

4.1.7. Bacterial content of the system

The bacteria added to the system to create the bacterioneuston were added to a final concentration of $\sim 6.5 \times 10^{10}$ cells per tank, this number were added to try to ensure enough activity to be noticeable in the short time available to the experiments.

All bacteria were added 12 hours before the start of an experiment, to allow homogenisation, acclimatisation and for any dissolved methane in the culture to be either removed by the bacteria (Table 2.1) or equilibrated with the atmosphere.

Homogeneity of the gas phase was ensured by the inclusion of two brushless miniature fans (RS Components), each with a capacity of ~ 12 litre air.min⁻¹. Mixing of the water phase and the production of the interfacial turbulence were by a baffle (46 x 12 x 0.6 cm), driven by a 12 V geared motor (RS Components). The baffle produced turbulence by pivoting at one edge (Figure 4.3) and moving through an arc of 8 cm.

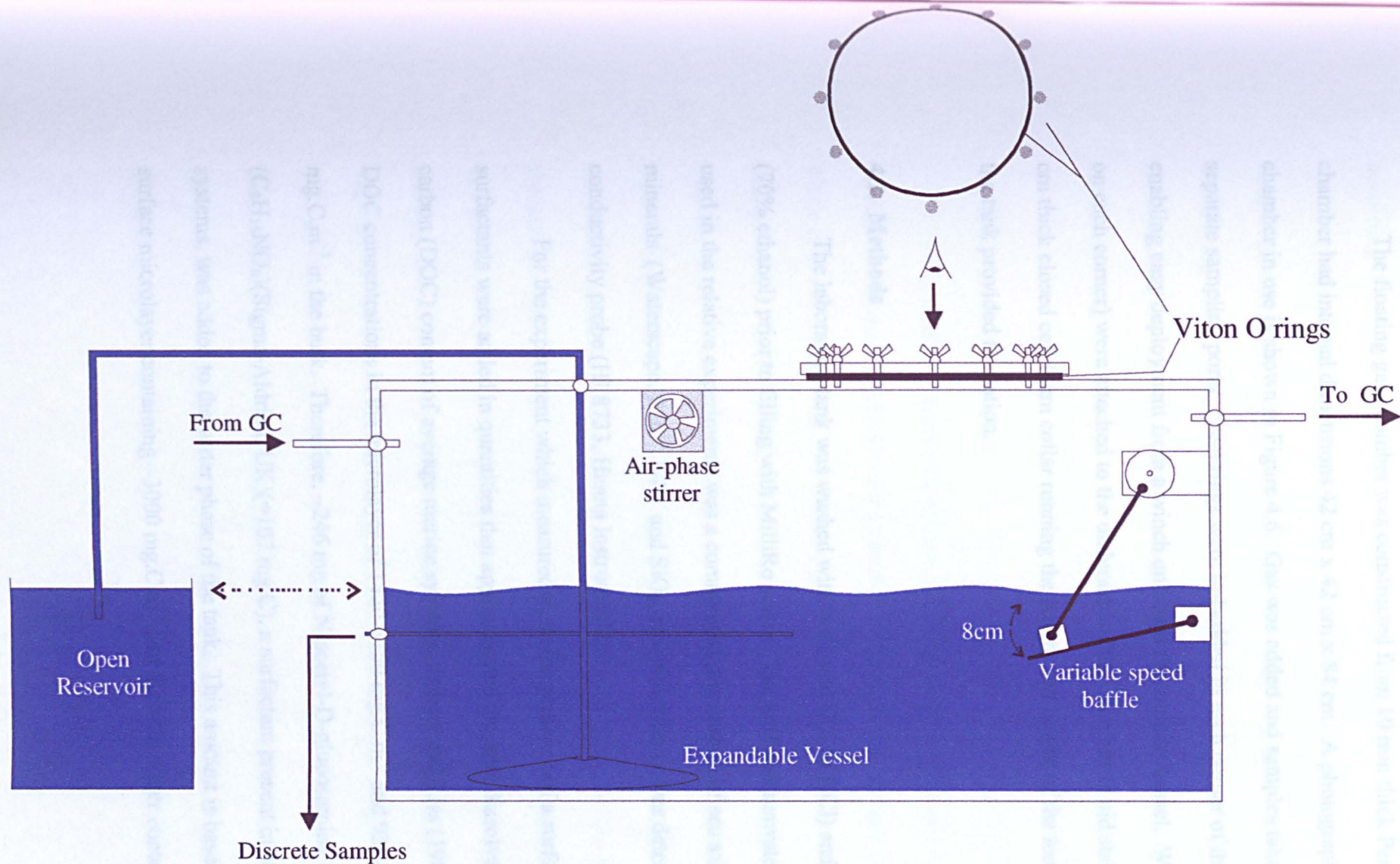


Figure 4.3

A schematic of the laboratory gas exchange tank. The expandable vessel has free connection to an open external reservoir and acts to maintain constant pressure inside the tank. Water in the open reservoir is held at the same level as the internal tank level.

4.1.8. Development of the floating gas chamber

The floating gas chamber was constructed from 10 mm thick Perspex. The chamber had internal dimensions 42 cm x 42 cm x 84 cm. A photograph of the chamber in use is shown in Figure 4.6. Gas was added and samples taken from separate sampling ports. Steel rings were imbedded in each corner of the chamber, enabling easy deployment from a winch on board the support vessel. Weights (3 kg on each corner) were attached to the underside of the chamber to aid stability. A 15 cm thick closed cell foam collar running the length and height of the longest side of the tank provided floatation.

4.2. Methods

The laboratory tank was washed with both acid (2.5 M HCl) and alcohol (70% ethanol) prior to filling with MilliRo water. The artificial seawater (ASW) used in the relative experiments was a commercial preparation of sea salt and minerals (Waterscape, PO_4^{3-} , NO_3^- and SiO_2 free) and salinity was determined with a conductivity probe (HI 8733, Hanna Instruments).

For the experiment which measured k_w in the presence of a surface film, surfactants were added in quantities that approximated the total dissolved organic carbon (DOC) content of average marine systems. Hunter and Liss (1981) reported DOC concentrations in the microlayer of 1420-3150 mg.C.m^{-3} and 920-1100 mg.C.m^{-3} in the bulk. Therefore, ~246 mg of N-acetyl-D-glucosamine ($\text{C}_8\text{H}_{15}\text{NO}_6$)(Sigma-Aldrich, UK)(=107 mg.C), a surfactant present in natural systems, was added to the water phase of the tank. This amount is based on a 50 μm surface microlayer containing ~3000 mg.C.m^{-3} and the bulk water containing ~1000

mg.C.m⁻³, presuming the surfactant partitions between the microlayer and the bulk according to the ratio suggested by Hunter and Liss (1981).

To ensure the tank water was in equilibrium with the laboratory air at the start of an experiment, laboratory air was circulated through the tank water via an aerator stone using a high delivery pump (Millipore, UK at >30 litre.min⁻¹) for ~12 hr. Before an experiment began, the tank was sealed and the *pp* checked to ensure equilibrium had been reached.

4.2.1. Invasive experiments

To initiate an invasive experiment, V12, V4 and V6 (Figure 4.2) were switched to mode (1) (green arrows) which connected the GC sample loops to the air phase of the exchange tank. An initiator gas was then injected into V5 and the circuit pump switched on for ~30 s to force the initiator gas into the headspace. The initiator gases were standards with predetermined mixing ratios (MR), so called because they were used to initiate experiments and to differentiate them from calibration standards. The air phase fans were turned on after the introduction of the initiator gases and the system left for ~5 min for air phase homogenisation. During this period, the movement of gas into the water was negligible. After air phase homogenisation, the circuit pump was switched on for the duration of the experiment. 80 ml of the CH₄ and N₂O initiator gases (comprising 1% CH₄ and 1% N₂O in N₂, respectively) and 30 ml of the SF₆ initiator gas (2.14 ppmv SF₆ in N₂) were added to the tank air phase at the start of an invasive experiment. After the addition of the initiator gases, the initial tank air phase *pp* were ~9 ppmv (CH₄), ~7.5 ppmv (N₂O) and ~570 pptv (SF₆).

The N₂O and CH₄ initiator gases were from a commercially available source (“Check Can”, Phasesep), filled by the manufacturer from a gravimetrically produced source gas (quoted accuracy 1%). The quoted filling tolerance was $\pm 5\%$, which gave an overall uncertainty of $1+0.06-0.04\%$.

4.2.2. Evasive experiments

To initiate an evasive experiment, a sample of gas exchange tank water was equilibrated with each of the three initiator gases and added back to the tank water. Three glass flasks (0.1 litre for CH₄, 0.2 litre for N₂O and 0.5 litre for SF₆) were filled with tank water, leaving ~ 0.02 litre headspace (Table 4.1). The headspace of each flask was replaced by one initiator gas, the flasks sealed and the two phases (water and headspace) were equilibrated by circulating the headspace through the water for 10 min. The equilibration apparatus is shown in Figure 4.4.

By calculating the partitioning of gas between the water and headspace after equilibration, the theoretical equilibrium partial pressure (P_{ge}) was found (Frost, 2000). The headspace must be flushed with fresh initiator gas (~ 3 times its volume) and re-equilibrated several times so that the P_{ge} equals the initiator gas partial pressure. After 5 flushings and re-equilibrations, P_{ge} equalled the initiator gas partial pressures for CH₄ and SF₆. Due to the solubility of N₂O however, the N₂O P_{ge} would only equal the N₂O initiator gas partial pressure after repeating this procedure >30 times. Therefore, a P_{ge} value of $\sim 80\%$ of the N₂O initiator gas pp was selected (Frost, 2000) which required the procedure of flushing and re-equilibration with N₂O initiator gas to be repeated ~ 11 times.

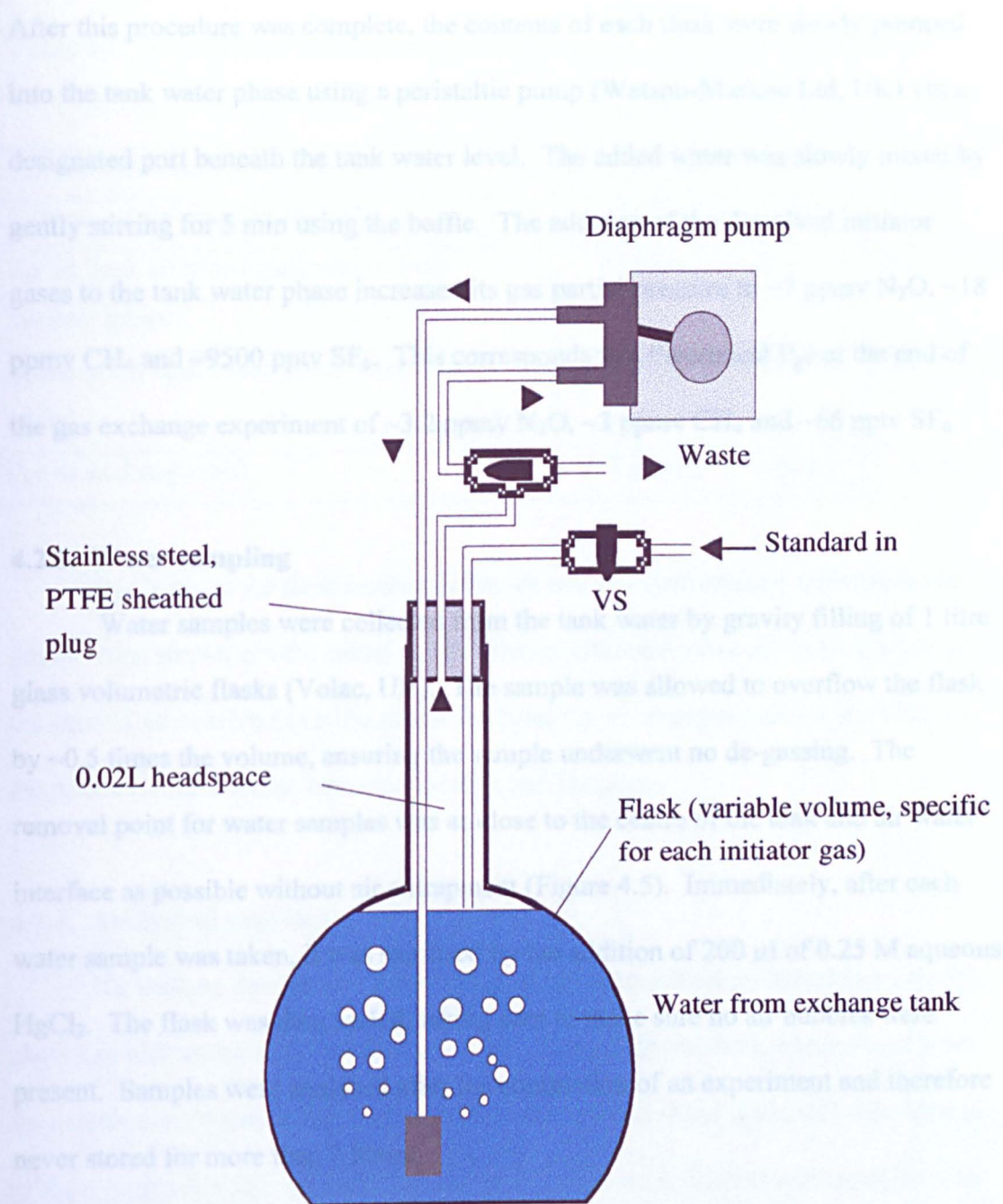


Figure 4.4 Apparatus used to equilibrate tank water with initiator gas

After this procedure was complete, the contents of each flask were slowly pumped into the tank water phase using a peristaltic pump (Watson-Marlow Ltd, UK) via a designated port beneath the tank water level. The added water was slowly mixed by gently stirring for 5 min using the baffle. The addition of the dissolved initiator gases to the tank water phase increased its gas partial pressure to ~7 ppmv N₂O, ~18 ppmv CH₄ and ~9500 pptv SF₆. This corresponds to a theoretical P_{ge} at the end of the gas exchange experiment of ~3.2 ppmv N₂O, ~3 ppmv CH₄ and ~66 pptv SF₆.

4.2.3. Water sampling

Water samples were collected from the tank water by gravity filling of 1 litre glass volumetric flasks (Volac, UK). The sample was allowed to overflow the flask by ~0.5 times the volume, ensuring the sample underwent no de-gassing. The removal point for water samples was as close to the centre of the tank and air water interface as possible without air entrapment (Figure 4.5). Immediately, after each water sample was taken, it was sterilised by the addition of 200 µl of 0.25 M aqueous HgCl₂. The flask was then sealed, taking care to make sure no air bubbles were present. Samples were analysed after the completion of an experiment and therefore never stored for more than 7 hours.

Table 4.1. Summary of volumes and partial pressures for evasion experiments.

	N ₂ O	CH ₄	SF ₆
Volume of water phase in flask (l)	0.1	0.2	0.5
Volume of air phase in flask (l)	0.02	0.02	0.02
P _{ge} in flask after re-equilibration with initiator gases	0.008 v	0.01 v	2.14 ppmv
Initial pp in tank water phase after flask added	7.1 ppmv	18 ppmv	9500 pptv
P _{ge} in exchange tank	3.2 ppmv	3 ppmv	66 pptv

P_{ge} is given for flask contents after several re-equilibrations with the initiator gases. Also shown are the initial *pp* for the experimental gases in the tank water at the start of an evasive experiment and the final P_{ge} of an experiment in the tank, i.e. the *pp* after equilibration between the tank and air phase.

4.2.4. Analytical overview

To analyse the discrete water samples, all valves were switched to mode (b), shown as red arrows and lines in Figure 4.2. Essentially the tank was isolated from the circuit via V4 and V6 and the equilibrator manifold (see Figure 4.5) was opened to the circuit via V12. All lines and sampling loops were flushed with equilibration gas (compressed air with pre-determined CH₄, N₂O and SF₆ mixing ratios close to ambient) prior to each analysis, in order to remove any traces of the previous sample. During analysis, a fixed volume headspace was created in the sample flasks by displacement of sample with equilibration gas. This headspace was then equilibrated with the water sample before analysis by GC, similar in principle to the procedure of Upstill-Goddard *et al.* (1996), but with some modification. For a more detailed overview, see Frost, 2000.

4.2.5. Analytical methodology

Flushing the circuit (all tubing and sample loops) with equilibration gas was achieved via V9, which opened >99% of the circuit to the equilibration gas via V11, the circuit pump was then switched on to speed flushing. The circuit was flushed for 2 min to remove traces of previous samples.

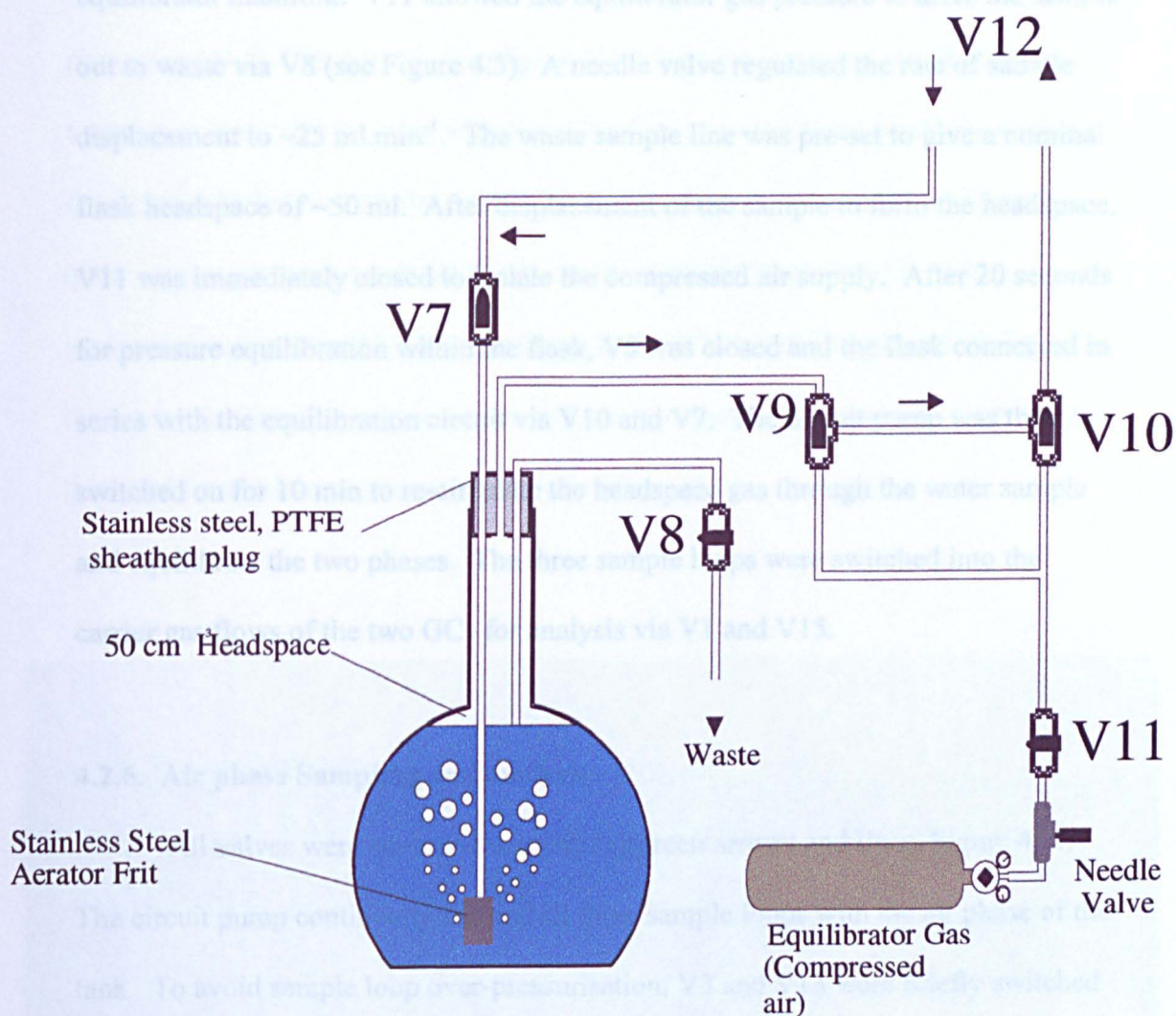


Figure 4.5.

Water sampling apparatus

Once flushing was completed, V11 was closed, V9 returned to original position and V10 reversed, V8 opened and V7 closed, this isolated the equilibrator system from the rest of the circuit, in preparation to receive a sample flask and create headspace (Figure 4.5).

After being kept at a constant temperature in a water bath during the course of the experiment (25 ± 0.5 °C) the seal of the sample flask was replaced with the equilibrator manifold. V11 allowed the equilibrator gas pressure to drive the sample out to waste via V8 (see Figure 4.5). A needle valve regulated the rate of sample displacement to $\sim 25 \text{ ml} \cdot \text{min}^{-1}$. The waste sample line was pre-set to give a nominal flask headspace of $\sim 50 \text{ ml}$. After displacement of the sample to form the headspace, V11 was immediately closed to isolate the compressed air supply. After 20 seconds for pressure equilibration within the flask, V8 was closed and the flask connected in series with the equilibration circuit via V10 and V7. The circuit pump was then switched on for 10 min to re-circulate the headspace gas through the water sample and equilibrate the two phases. The three sample loops were switched into the carrier gas flows of the two GCs for analysis via V1 and V15.

4.2.6. Air phase Sampling and analysis

All valves were switched to mode (a) (green arrows and lines, Figure 4.2.) The circuit pump continually flushed all three sample loops with the air phase of the tank. To avoid sample loop over-pressurisation, V3 and V13 were briefly switched (3s) to equilibrate the sample loops to laboratory pressure, prior to injection of the sample loop contents, via V1 and V15 into the GC carrier gas flow for analysis.

For a more detailed description of all the procedures, development and theory behind this part of the study see Frost, 2000.

4.2.7. Floating Gas Chamber Experiments

To validate any results from the laboratory tank, flux measurements must be made across an *in situ* marine surface microlayer. Preliminary experiments were conducted from a small Zodiac inflatable ~10 km offshore in the North Sea (~55°08'N 01°16'W) using a floating gas exchange chamber (Figure 4.6.). Within the chamber, gas flux was forced by augmenting the chamber gas partial pressure to be either greater than the underlying water (by increasing the chamber partial pressure of N₂O, CH₄ and SF₆ to a factor of >5 than the water) or less than the underlying water (by flushing with N₂). The flux was monitored by taking periodic duplicate chamber air samples (every 30 min) in gas tight glass syringes (stored under iced water in an insulated chest), which were analysed with the same equipment and that used for the laboratory tank. Water samples were taken by Niskin bottle from the support vessel and again analysed with the same equipment as that used for the laboratory gas exchange tank.

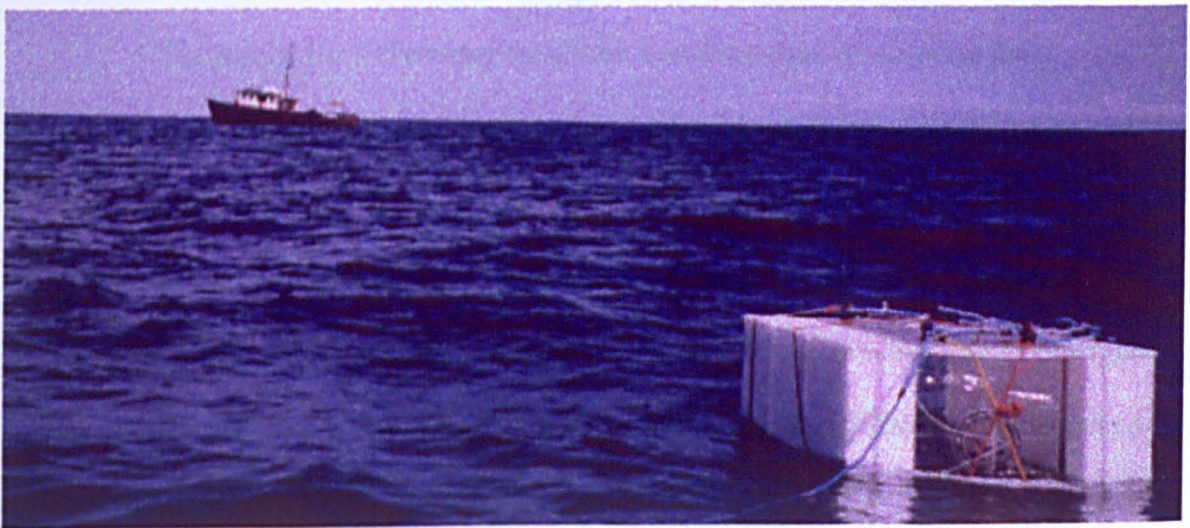


Figure 4.6. The floating gas chamber with the RSV *Bernicia* in the background

4.2.8. Gas chromatography

Chromatographic separation of equilibrated flask headspace and tank air phase gases was by isothermal gas chromatography at 60 °C. For CH₄ and N₂O, separation was on 2 m x 2 mm i.d. and 5 m x 2 mm i.d. 'Porapak-Q' (80-100 mesh) columns respectively. Separation of SF₆ was on 5 m x 2 mm i.d. 'Molecular Sieve' (5A, 80-100 mesh). All separations used UHP N₂ as carrier gas (BOC, >10 ppmv total gas impurities), pre-dried on a molecular sieve / activated charcoal filter and passed through an oxygen trap (Supelco). CH₄ was detected by a Flame Ionisation Detector (FID), N₂O and SF₆ were detected by an Electron Capture Detector (ECD).

Control of the Shimadzu GC and integration of N₂O and CH₄ peaks was by the Shimadzu 'Easychrom' software. SF₆ peaks were integrated on a Shimadzu CR6-A integrator. Instrument drift was undetectable over the time periods of the experiments (maximum 3 days) and calibration curves were produced from the standard gases for both GCs for each tank gas exchange experiment.

4.3. Results

The results for these experiments are best illustrated by showing the difference in transfer velocity, between those experiments using methanotrophs and those not, graphically.

These data are shown in a more complete and comprehensive manner in Frost, 2000. For the purposes of this part of the study the following figures illustrate the hypothesis. The methanotrophic species used in these experiments are described in more detail in Chapter 5.

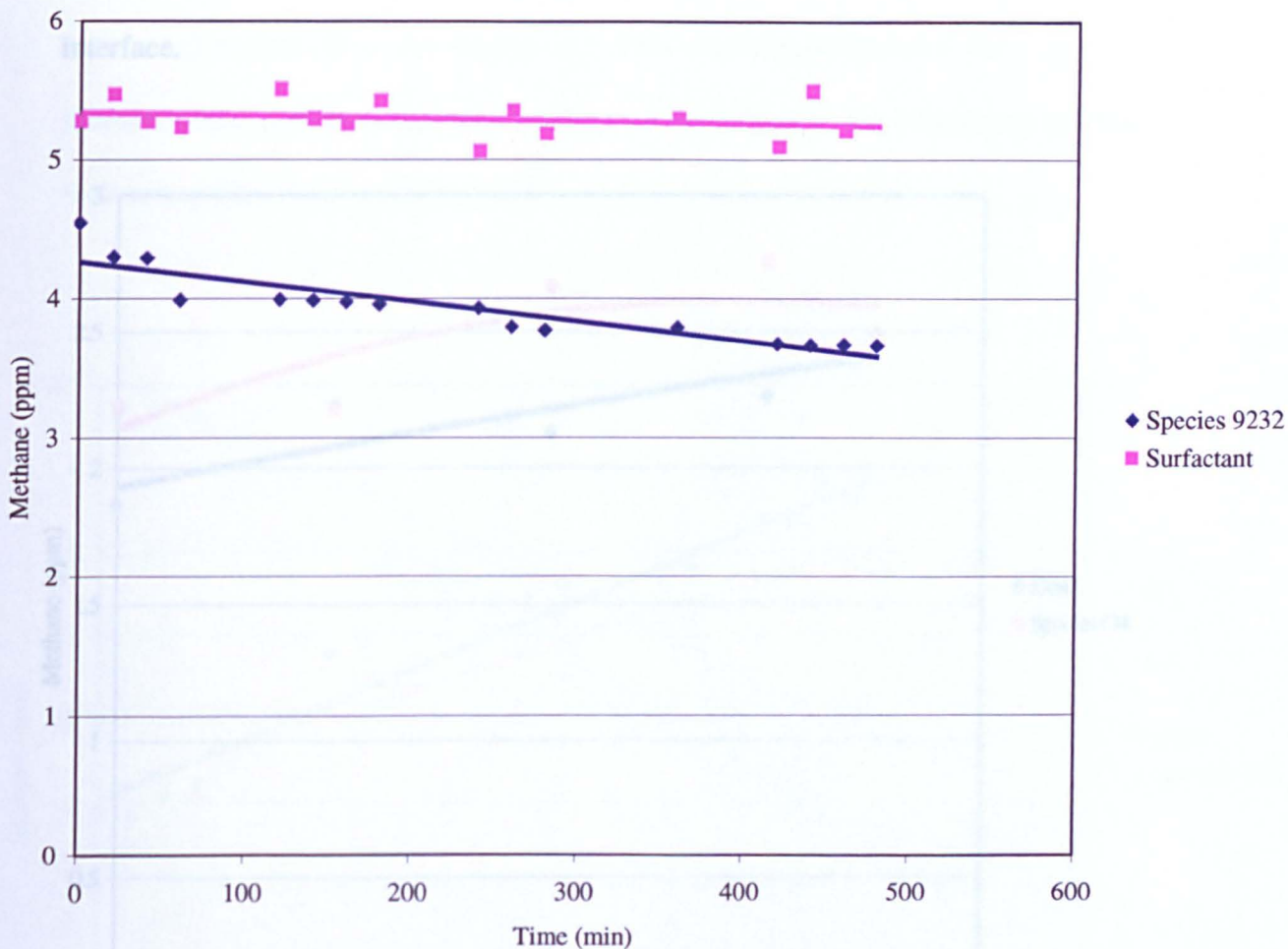


Figure 4.7. Concentration of CH_4 in the headspace of the tank over the time course of an invasive experiment comparing water seeded with the methanotroph 9232 and surfactant

What can clearly be seen is the effect the presence of a methanotroph in the water body has on the transfer velocity of the methane.

The presence of the surfactant mimics the effect of a biological layer on the surface, enabling us to credit the difference in methane concentration to the methanotrophs added. That is, the only difference between the two systems is the presence of a methane oxidising organism, assuming the surfactant does reflect the properties of the biological layer. In particular, in this case, the formation of a

bacterioneuston, containing methanotrophs, that oxidises the methane at the surface interface.

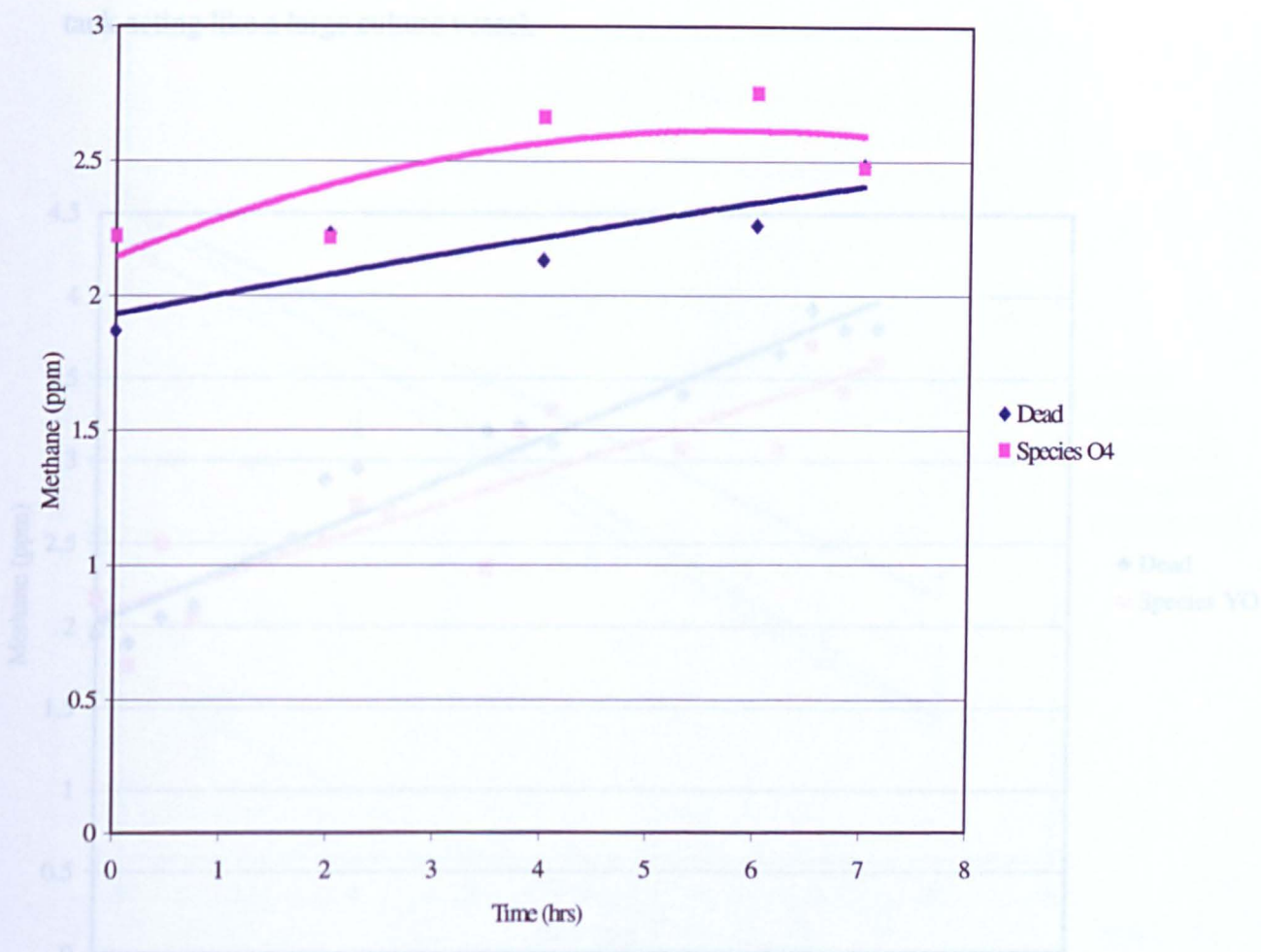


Figure 4.8. Concentration of CH₄ in the water phase from an invasive experiment, comparing methanotroph species O4 and methanotrophs which have been killed

Figure 4.9. Concentration of CH₄ in the brackwater during an invasive experiment comparing

Figure 4.8 complements Figure 4.7 in that we can now see the effect of a methanotroph community on the water phase of the tank. The figure demonstrates the increase in methane concentration of the water comparing the results from the experiment with species O4 and those from species YO plus mercuric chloride. The addition of HgCl₂ was to kill the methanotrophs in the tank so as to create a control. The figure shows the same initial increase in methane concentration to the water

phase but then the rate of increase in the concentration declines indicating the removal of methane from the water phase. This would be attributed to the methanotrophs present in the water phase, as well as those in the microlayer, and the tank acting like a large culture vessel.

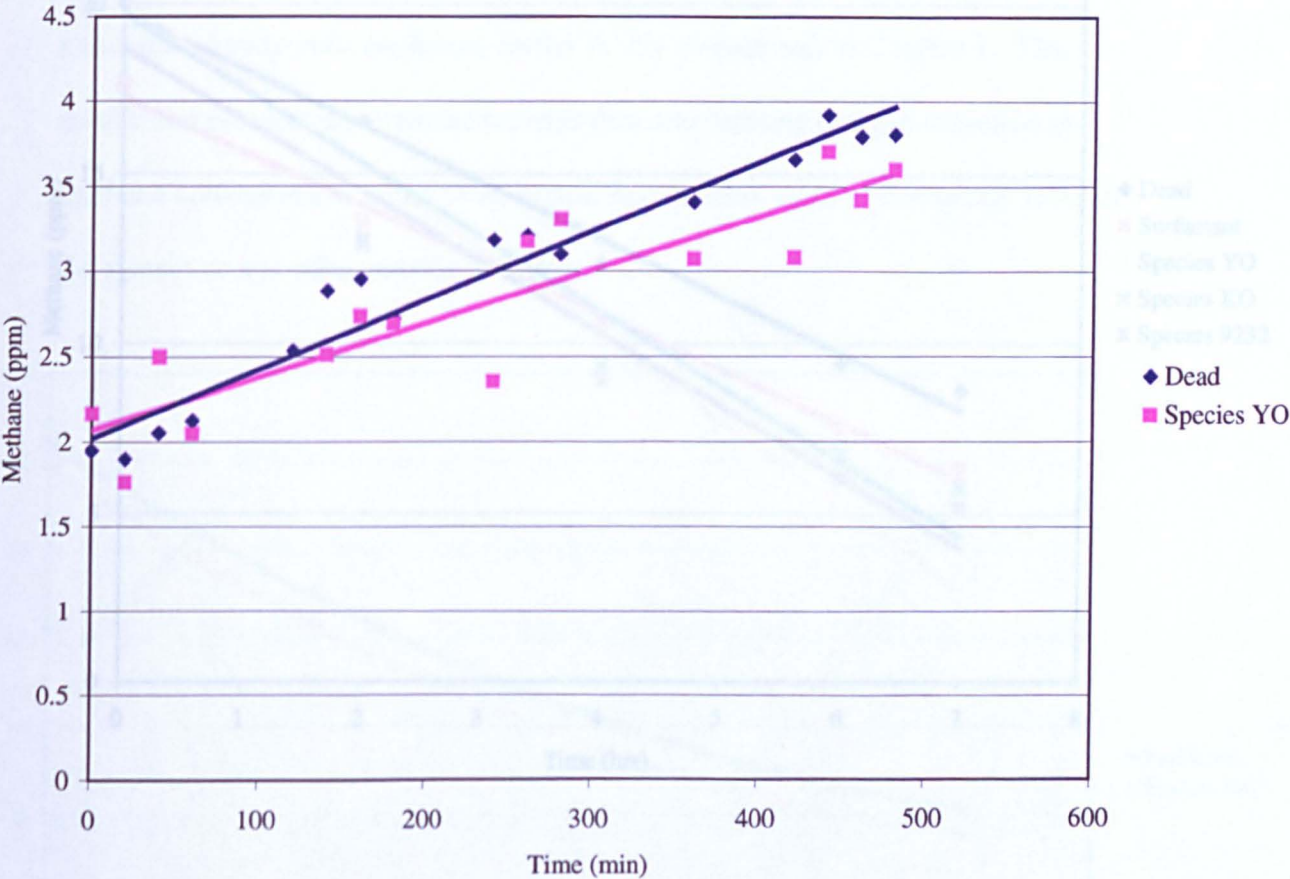


Figure 4.9. Concentration of CH_4 in the headspace during an evasive experiment compareing methanotroph species YO and Methanotrophs which have been killed

The complement to the previous graph can be seen in Figure 4.10. This figure also supports the assumption that the surfactant replicates a biological surface layer and the dead cells are biologically inert. Comparing the slopes of the two conditions, it is obvious they have a similar effect and when compared to slopes of known methanotrophs it can be seen that they have little or no effect on the transfer

methane and/or methanotrophs forming a bacterioneuston and oxidising the methane at the interface.

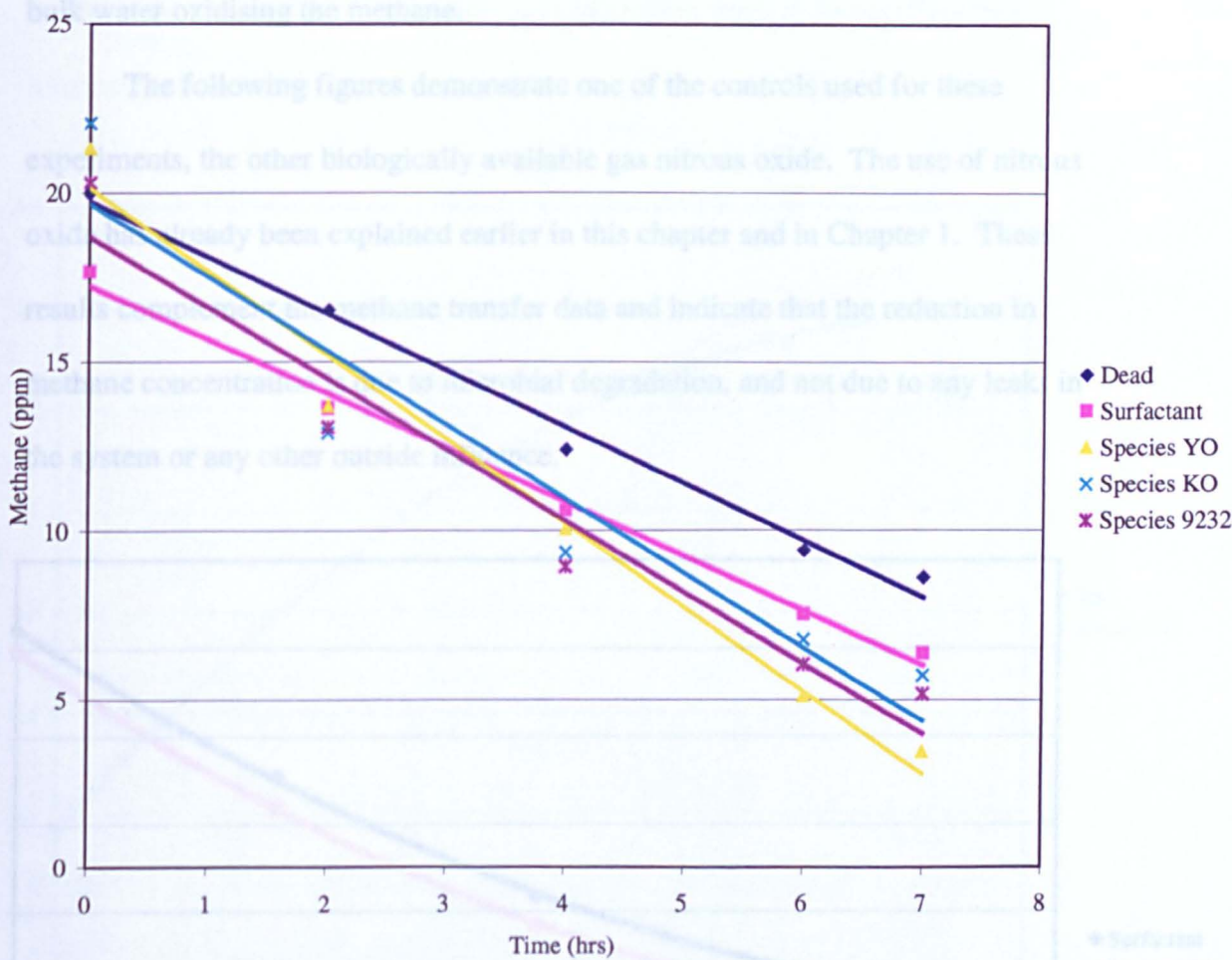


Figure 4.10. Concentration of CH_4 in the water phase during an evasive experiment comparing three methanotroph species, methanotrophs which have been killed and surfactant

The complement to the previous graph can be seen in Figure 4.10. This figure also supports the assumption that the surfactant replicates a biological surface layer and the dead cells are biologically inert. Comparing the slopes of the two conditions, it is obvious they have a similar effect and when compared to slopes of known methanotrophs it can be seen that they have little or no effect on the transfer

rate of methane between the two environments. Figure 4.10 also shows the decrease in methane concentration in the bulk water with the addition of methanotrophs, this, however, proves nothing new as it could just be the methanotrophs present in the bulk water oxidising the methane.

The following figures demonstrate one of the controls used for these experiments, the other biologically available gas nitrous oxide. The use of nitrous oxide has already been explained earlier in this chapter and in Chapter 1. These results complement the methane transfer data and indicate that the reduction in methane concentration is due to microbial degradation, and not due to any leaks in the system or any other outside influence.

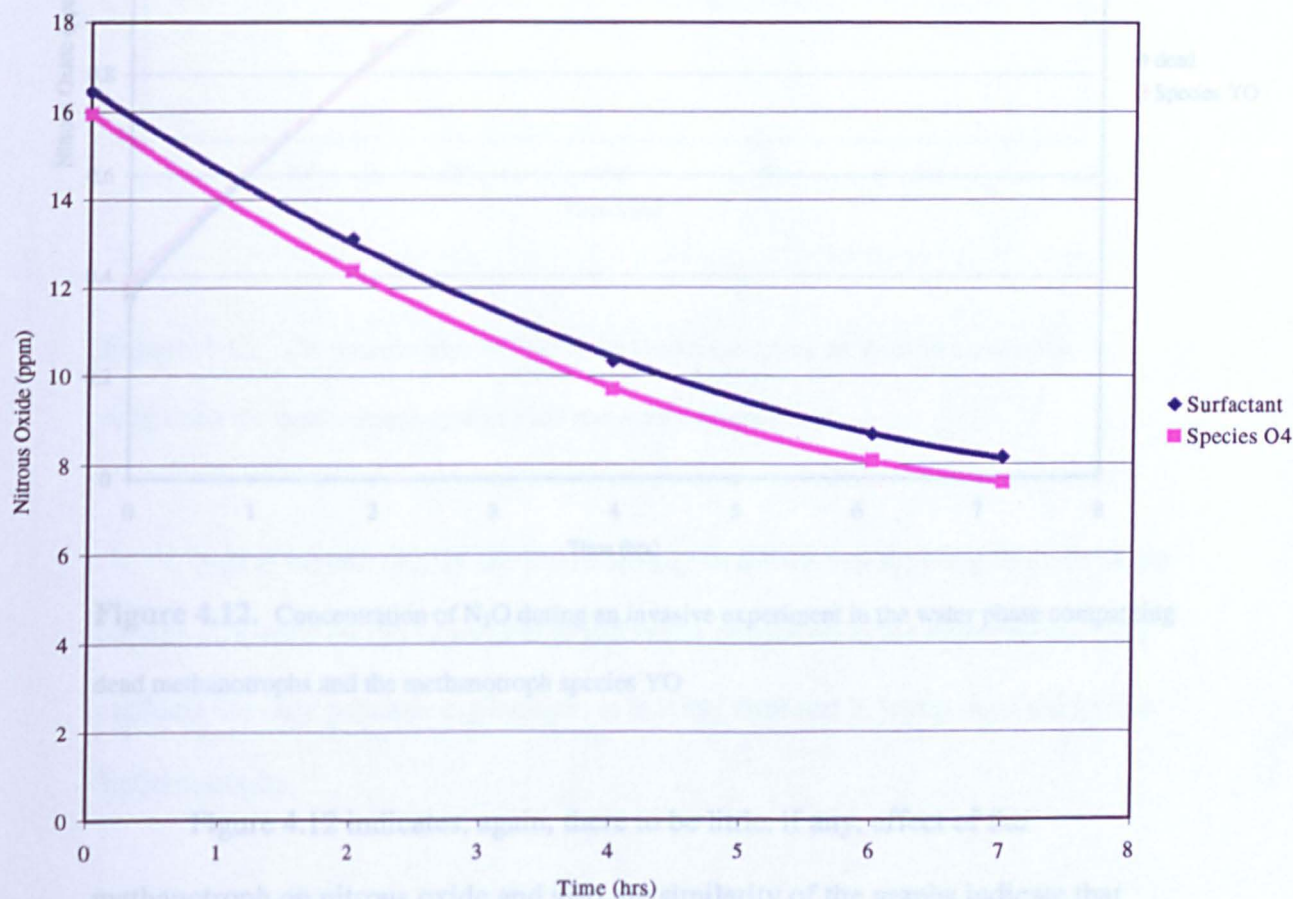


Figure 4.11. Concentration of N_2O in the water phase during an evasive experiment comparing surfactant and the methanotroph species O4

The figure (4.11) above shows the rate of transfer of nitrous oxide between the water and the headspace comparing the experiment using the methanotroph species 04 and the surfactant. The figure shows the rate of transfer of the gas, nitrous oxide, to be approximately the same, thus indicating there to be no effect by the microorganism on the nitrous oxide.

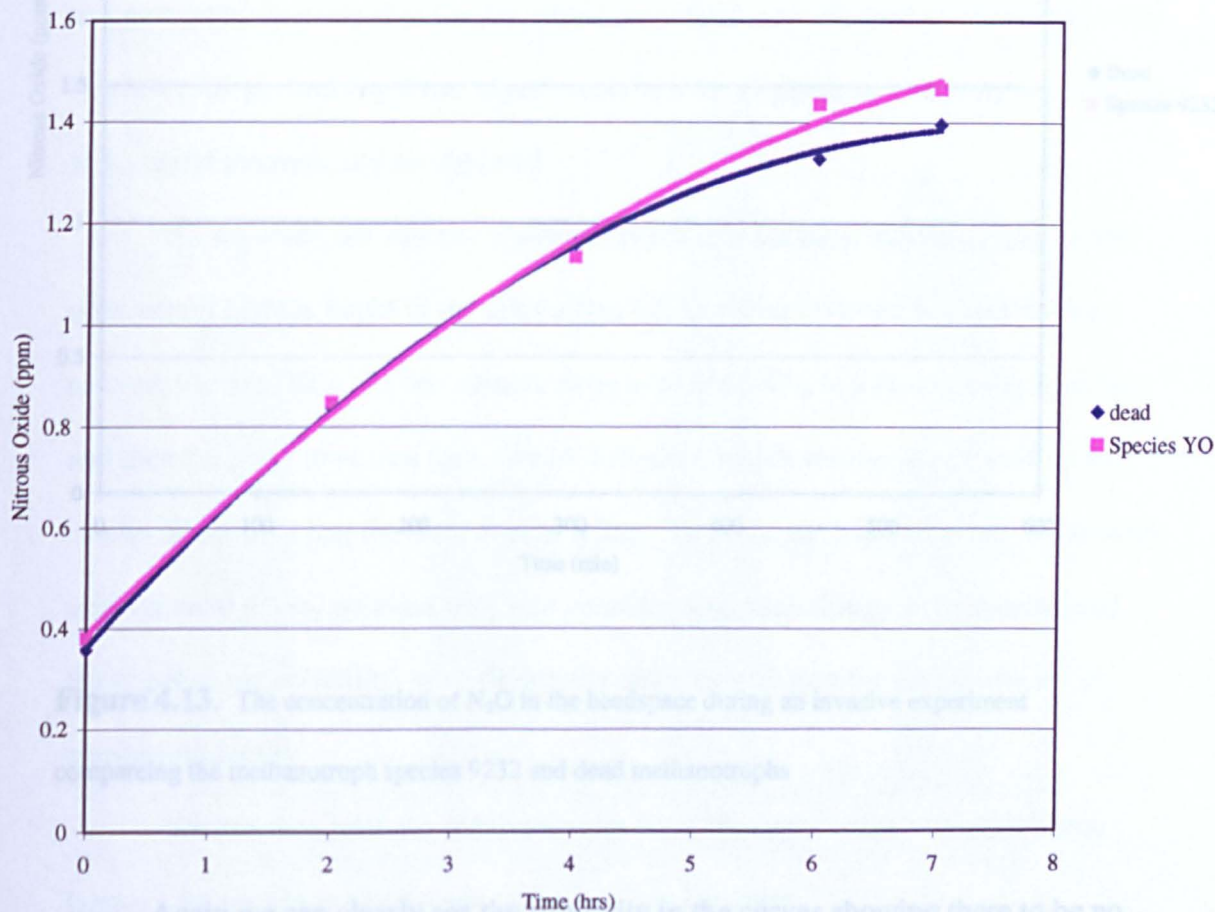


Figure 4.12. Concentration of N_2O during an invasive experiment in the water phase comparing dead methanotrophs and the methanotroph species YO

Figure 4.12 indicates, again, there to be little, if any, effect of the methanotroph on nitrous oxide and also the similarity of the graphs indicate that there is no leakage in the system.

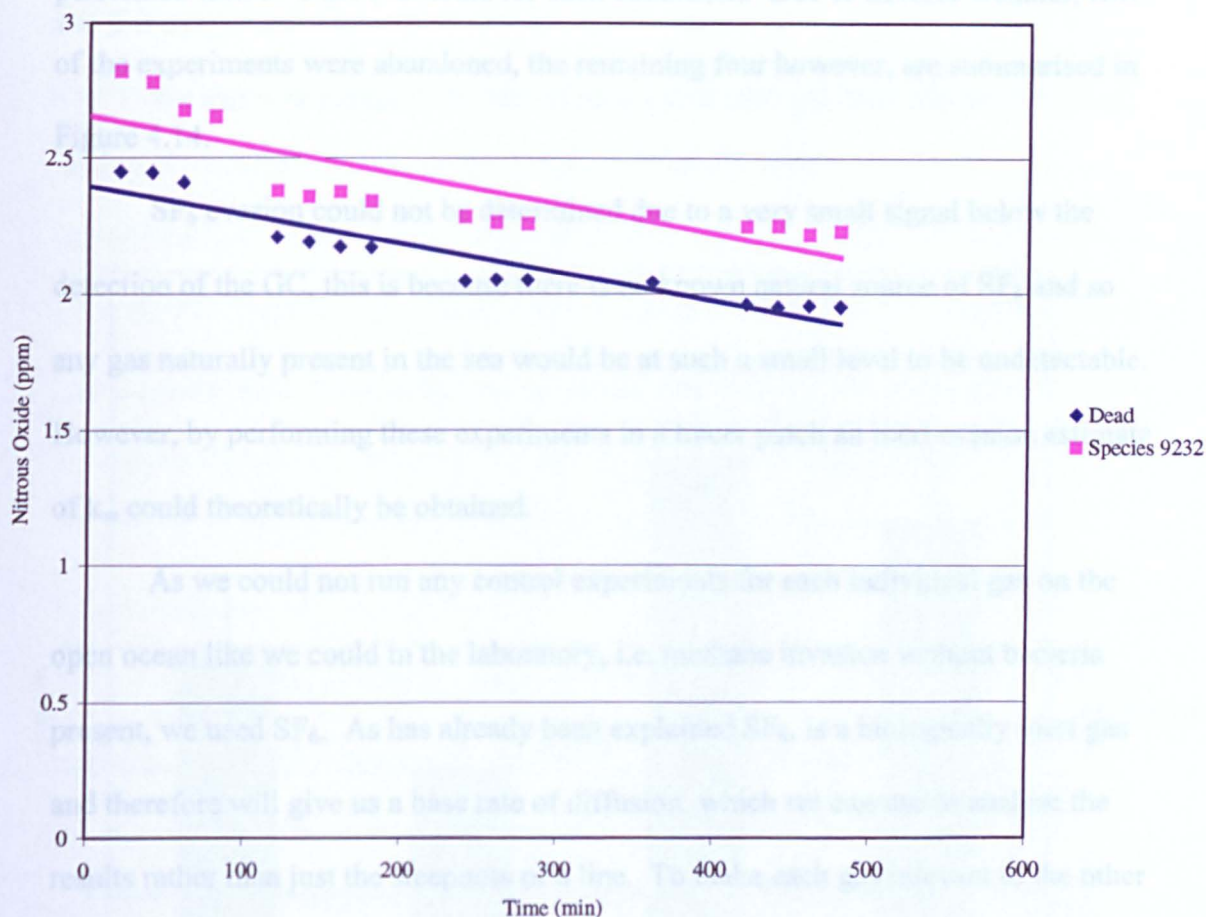


Figure 4.13. The concentration of N_2O in the headspace during an invasive experiment comparing the methanotroph species 9232 and dead methanotrophs

Again we can clearly see the similarity in the curves showing there to be no outside influence on the experiments and when compared to the experiments with methane the only possible explanation is that the methane is being oxidised by the methanotrophs.

4.3.1. *In situ* gas measurements

Deployment of the floating gas chamber required calm conditions and therefore put constraints on the work. However, the six experiments that were

conducted consisted of both invasive and evasive parts. Each set of experiments performed lasted ~5 hrs (~2 hours for each condition). Due to adverse weather, two of the experiments were abandoned, the remaining four however, are summarised in Figure 4.14.

SF₆ evasion could not be determined due to a very small signal below the detection of the GC, this is because there is no known natural source of SF₆ and so any gas naturally present in the sea would be at such a small level to be undetectable. However, by performing these experiments in a tracer patch an inert evasion estimate of k_w could theoretically be obtained.

As we could not run any control experiments for each individual gas on the open ocean like we could in the laboratory, i.e. methane invasion without bacteria present, we used SF₆. As has already been explained SF₆ is a biologically inert gas and therefore will give us a base rate of diffusion, which we can use to analyse the results rather than just the steepness of a line. To make each gas relevant to the other experimental gases, we must take into consideration such things as temperature of the experiment, solubility, specific gravity and Schmidt number and create a conversion factor.

We can then take our measurements from the experiment and apply these factors. So we apply our figures to the gas flux equation:

$$K_{w-gas} = B_E \cdot k_w$$

Where

$$B_E = \frac{F_{gas}}{k_w(C_w - \beta \cdot C_a)}$$

F is the conversion factor for each gas.

Where C_w is concentration in the water, C_a is concentration in the atmosphere and β is a constant.

We can now compare the rate of removal of each gas from the subject phase.

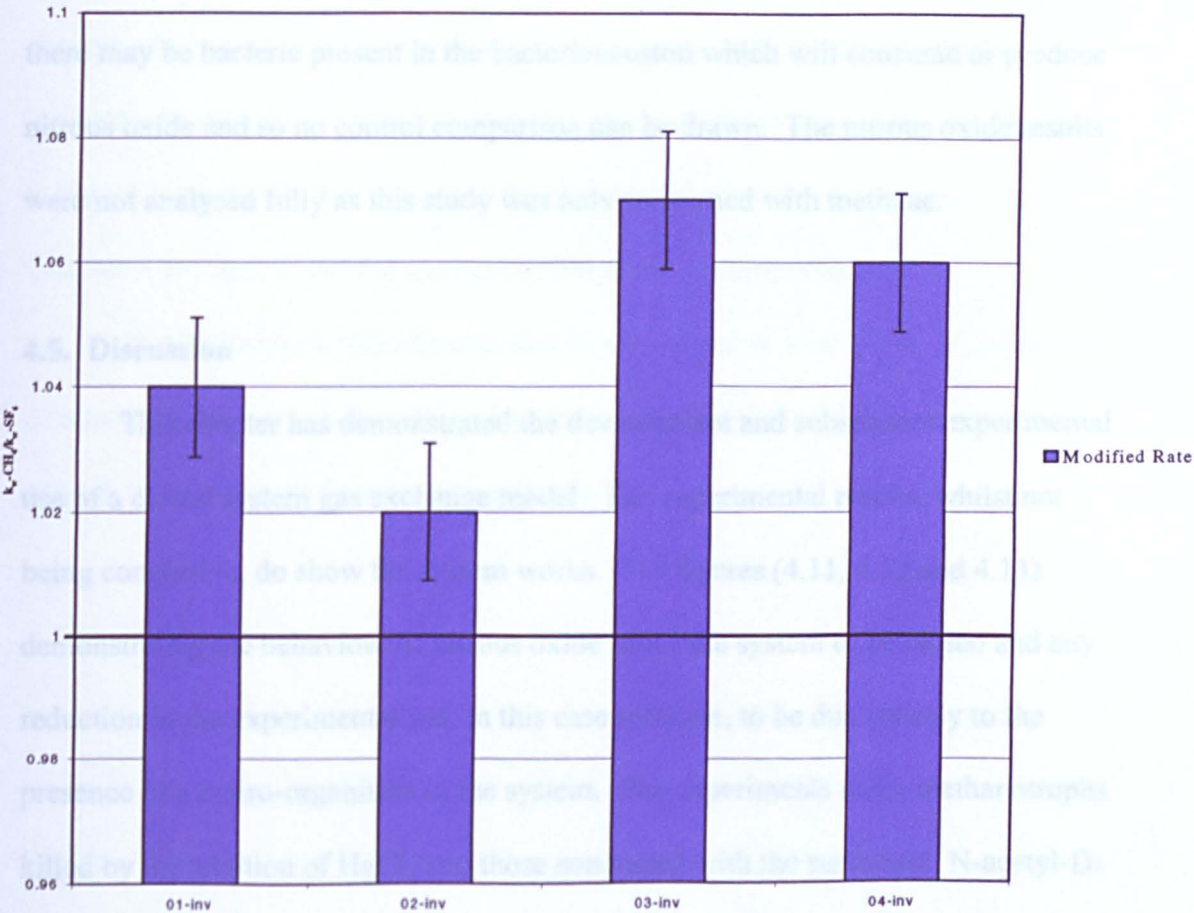


Figure 4.14. k_{w-CH_4}/k_{w-SF_6} ratios for the invasive *in situ* experiments

We can now infer as to whether or not there is an influence upon the removal of methane from the subject phase which is not applied to the sulphur hexafluoride. That is, a biological component within the system that increases the rate of removal of methane from the phase in question, in this case the atmosphere. The increase can be seen in Figure 4.14 where the respective rates of gas transfer for SF_6 and CH_4 are

divided, showing here where the rate of removal for CH_4 is divided by the rate of SF_6 removal. The results show that the rate of removal of methane is higher, all other factors being equal, and so we can infer that there must be a factor affecting the methane which does not effect the SF_6 , i.e. methanotrophs present in the bacterioneuston. Nitrous oxide controls are not valid for methane consumption as there may be bacteria present in the bacterioneuston which will consume or produce nitrous oxide and so no control comparison can be drawn. The nitrous oxide results were not analysed fully as this study was only concerned with methane.

4.5. Discussion

This chapter has demonstrated the development and subsequent experimental use of a closed system gas exchange model. The experimental results, whilst not being conclusive, do show the system works. The figures (4.11, 4.12, and 4.13) demonstrating the behaviour of nitrous oxide prove the system to be closed and any reduction in the experimental gas, in this case methane, to be due entirely to the presence of a micro-organisms in the system. The experiments using methanotrophs killed by the addition of HgCl_2 and those conducted with the surfactant, N-acetyl-D-glucosamine, demonstrate that any difference in transfer rate from one phase to another must be due to the controlled addition of methanotrophs and not a change in the properties of the surface microlayer. The most interesting result is the rate of reduction of methane from the headspace in the invasive experiment (Figure 4.7). This increased rate of gas transfer from the headspace can only be due to an effect from the surface microlayer as it is this interface which controls the exchange. The other results cannot be attributed solely to a microlayer effect, as there will be methanotrophs present in the bulk water phase. This means any methane in the water

will be as liable to oxidation by a methanotroph present in the bulk phase as one present in the microlayer.

The data from the floating gas chamber work are just preliminary and needs further investigation but the methodology has been shown to be consistent and reliable. The first result from using this equipment show there to be an increase in the rate of removal of methane when compared to that of sulphur hexafluoride. This indicates the presence of viable methanotrophs in the bacterioneuston. During this study we have, in Chapter 3, proved the presence of a bacterioneuston, and, in Chapter 5 we have identified methanotrophs in the bacterioneuston.

Further work on this area can also be carried out to look at the rate of removal of nitrous oxide and any bacteria present which may assist this but there was not enough time in this study to investigate fully.

Chapter Five

Methanotrophs in the sea surface microlayer

5.1. Introduction

Recent advances in the application of molecular genetic approaches have emphasised the potentially huge underestimate of microbial diversity in a range of natural environments. These approaches, however, give no direct information about the biogeochemical processes in which microorganisms are active. This chapter coupled with Chapter 4 tries to tie two areas within this study together.

One of the ultimate aims of this study was to identify methanotrophs within the hydrosphere, in particular the bacterioneuston. This was done once the gas exchange results using the *in situ* floating gas exchange tank had been established. Another aim was to increase our knowledge of methanotrophs in marine environment, using molecular methods.

5.1.1. Methane in the sea

Interactions of CO_2 and CH_4 between water and the atmosphere are key to understanding the biological and chemical dynamics of bodies of water, whether they be lakes or oceans (Striegl and Michmerhuizen, 1998). CO_2 assimilated by the hydrospheres productivity can be input directly from external sources, such as atmospheric CO_2 and dissolved CO_2 in ground water and surface water, or internally by biological and chemical transformation of inorganic and organic carbon.

A relatively small proportion of CH_4 dissolved in the marine environment is input from external sources. Although some CH_4 may be dissolved in surface water inflow or may mix from the atmosphere to CH_4 depleted water, the majority of dissolved CH_4 in temperate water bodies is produced by anaerobic decomposition of organic matter in sediments. This CH_4 moves upward to the water column, where it

is ultimately converted to biomass and CO₂ by methanotrophic bacteria or emitted to the atmosphere (Lidstrom and Somers, 1984).

Methane can also be found in gas hydrates. These are crystalline compounds composed of water molecules that form rigid cage-like lattice structures, most of which are occupied by gas molecules (Bidle *et al.*, 1999). Much of the current interest in gas hydrates stems from the fact that they are estimated to represent the largest hydrocarbon fuel reservoir on Earth, pose a potential geohazard in submarine slopes, and may be a source of atmospheric methane. The stability of gas hydrates depends on low temperature, elevated pressure, and the appropriate chemical environment. Therefore, the distribution of gas hydrates is primarily restricted to polar and deep oceanic regions.

Holmes *et al.*, (2000) showed that methane concentrations were supersaturated (up to 90%) with respect to the atmosphere in the upper 300 m in both the North Pacific and North Atlantic. De Angelis and Scranton (1993) stated that microbial CH₄ oxidation is the only process occurring within the water column that could prevent eventual loss of CH₄ to the atmosphere.

Methanotrophic bacteria oxidise CH₄ to CO₂ under aerobic conditions, methane oxidation also occurs under anaerobic conditions, but up until 1999 the organisms responsible for, and the biochemistry of, this process were not known (Joye *et al.*, 1999). However recent work by Orphans *et al.*, (2001a,b) and Hinrichs *et al.*, 1999 have shed a lot more light on this area.

5.1.2. Methanotrophs in the sea

Over the past three decades, methanotrophic bacteria have been studied in the laboratory or in industrial applications, and a number of factors controlling their

activity have been examined in detail in pure and mixed cultures (Dumestre *et al.*, 1999). These experiments have led to an understanding of how methanotrophs may operate in natural environments, such as termite mounds, lake water, sediments and soils. One environment, which has not been investigated, is the sea surface microlayer. Within this layer exists a community of bacteria known as the bacterioneuston (see Chapter 3) which may contain methanotrophs.

As all methanotrophs possess a membrane-bound monooxygenase (particulate methane monooxygenase), and some also possess a soluble, cytoplasmic enzyme complex (soluble methane monooxygenase) the specific genes coding for these can be used as biomarkers (see sections 1.13 and 1.14).

Part of this study was designed to identify any possible methanotrophs within this community.

5.2. Materials and methods

5.2.1. Study site

The sample area was started from 55°08'N 01°16'W, which is approximately 10 km off the coast from Blythe, UK.

5.2.2. DNA extraction

DNA was extracted from the samples of pelagic water and sea surface microlayer as described in Chapter 2, Materials and Methods (section 2.10). The same DNA stocks were used as in Chapter 3.

5.2.3. PCR, cloning and sequencing

The *pmoA* specific primers used were mb661 in conjunction with A189gc (see Table 2.2), which an internal piece of *pmoA* of approximately 470 bp.

The primers, which are specific for part of the *mmoX* gene used to identify the presence of sMMO, were X536f and X898r. The amplification using these primers yielded a product of approximately 360 bp. Another set of *mmoX* specific primers were also used, *mmoX* 882f and *mmoX* 1403r (Table 2.2), to analyse a less conserved area of the gene. These primers yielded a product of approximately 520 bp.

5.2.4. RFLP analysis

Clones were chosen at random and the plasmid isolated by alkaline lysis (Chapter 2). Plasmids were digested with restriction enzymes. Fragments were resolved on 2% agarose and grouped manually into operational taxonomic units (OTUs) (Moyer *et al.*, 1994) based on their restriction patterns.

5.2.5. Sequence analysis

Analyses and translation of DNA-derived polypeptide sequences were carried out using the Genetics Computer Group (GCG) programs (Wisconsin) and the ARB package (Section 2.15).

5.2.6. Marine methanotrophs

The marine methanotrophs IR1 and DR1, which were identified by Lees *et al.*, 1991, kept in the culture collection at Warwick University could not be recovered and other marine methanotrophs were not available.

5.3. Results

5.3.1. *pmoA* sequences

Using PCR primers specific for the detection of methanotrophs from the DNA collected from the two environments, the sea surface microlayer and the pelagic waters, it is possible to comment on the methanotroph assemblage of the bacterioneuston. Section 3.3.1 demonstrates the ability of the sample method to only collect DNA from the environment.

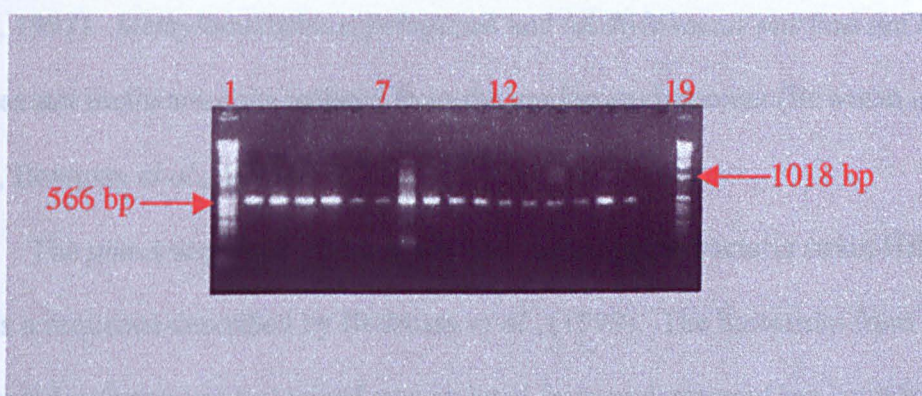


Figure 5.1. *pmoA* PCR amplification using primers A189gc and mb661r.

Lanes 1 and 19 are 1 kb DNA Ladder (molecular mass markers), 2-7 are replicates of DNA from pelagic water, 8-11 positive controls (known DNA), 12-17 DNA isolated from a bacterioneuston sample, 18 negative control (no template DNA).

The individual PCR amplification products from each environmental sample (bacterioneuston or pelagic) were pooled to try to lessen the effects of PCR bias.

Pooled PCR products were cloned using the TA cloning kit (Invitrogen) as described in Chapter 2.

A total of 200 clones (100 from the bacterioneuston library, 100 from the pelagic water library) were subjected to RFLP analysis. The 200 clones fell into 2 OTUs, OTU 1 contained all but two of the clones. OTU 2 contained only 2 two clones both from the pelagic library.

Random clones from within OTU 1 were sequenced, and found to be closely related with those *pmoA* sequences from *Methylobacterium pelagicum* (Sieburth *et al.*, 1987) (Figure 5.2). Both clones from OTU 2 were sequenced and found to be grouped with the derived *pmoA* amino acid sequence from the thermophilic bacteria HB (Bodrossy *et al.*, 1999).

The grouping of all the *pmoA* clones with *pmoA* sequences from the Type I methanotrophs is not unexpected, since marine environments have been shown to be dominated by a limited group of Type I methanotrophs (Holmes *et al.*, 1995b, Smith *et al.*, 1997). *Methylobacterium pelagicum* and *Methylobacter marinus* are also the only extant methanotrophs isolated from the marine environment (Bowman *et al.*, 1995, Bowman *et al.*, 1993).

The *pmoA* sequence which is labelled thermophilic bacteria strain HB (Figure 5.2) is a sequence described by Bodrossy *et al.*, (1999). The bacterium from which this *pmoA* sequence was obtained was isolated from underground hot springs in Hungary. The strain grew on methane at up to 72⁰C, the highest recorded growth for a methanotroph. 16S rDNA phylogenetic analysis showed strain HB to be a representative of a novel, deep branching group of the γ -Proteobacteria quite distinct from extant methanotrophs.

That the *pmoA* clones from the marine environment fell into only two OTUs was not a surprise because so far there have only been two species of methanotroph isolated from the marine environment and so the sequences might have been expected to be very similar. Costello and Lidstrom (1999) found that for *pmoA* clones obtained using the primers A189gc and mb661r, RFLPs could be observed but these would not differentiate between anything lower than species level and were not genus specific. Thus, looking at the dendrogram produced from this study (Figure 5.2) it is not surprising that even though there is some sequence variation, the two OTUs identified only allowed separation of the clones at genus level.

5.3.2. Soluble methane monooxygenase sequences

PCR amplification of *mmoX* from both the pelagic and the bacterioneuston DNA samples was successful using the primers X536f and X898r but not using the primers *mmoX* 882f and *mmoX* 1403r. This may be because the set of primers, X536f and X898r (Fuse *et al.*, 1998) were designed against a region of greater conservation, i.e. based on *mmoX* sequences from 4 genera including a, Type I methanotroph. In contrast the McDonald *et al.*, (1997), primers were only based on *mmoX* sequences from *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* OB3b, as these were the only full *mmoX* sequences available at the time.

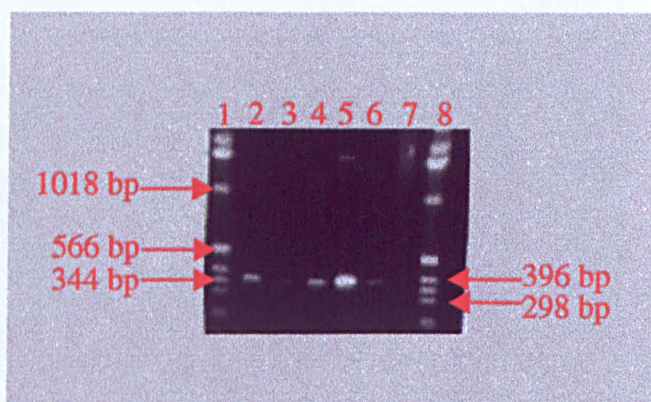


Figure 5.3. Amplification of the bacterioneuston and pelagic samples by the *mmoX* specific primers X536f and X898r, amplified product ~360bp.

Lanes 1 and 8 were 1 kb DNA Ladder (molecular mass markers), 2 and 3 were DNA from the bacterioneuston, 4 and 5 were DNA from Pelagic water, 6 Positive Control (*Methylosinus trichosporium* (Bath)) DNA, 7 Negative control (no template).

A total of 50 clones (with inserts) from both the pelagic and bacterioneuston libraries were chosen for analysis. The plasmids were digested with restriction enzymes. RFLP analysis of random clones identified one OTU, a number of clones were chosen for further analysis by sequencing. The single OTU had been expected

as McDonald *et al.* (1997) digested a number of sMMO genes with several restriction enzymes and all showed the same digestion pattern.

The *mmoX* sequences obtained were translated into their amino acid sequences, and aligned with reference sequences, Figure 5.4. The portion of the *mmoX* gene that was amplified is extremely conserved in all the *mmoX* sequences from extant methanotrophs as well as the environmental *mmoX* clones.

194	204	214	224	234	244	254	264	274	284	294	299	
SGDAVECSINLQLVGEACFTNPLIVAVTEWASANGDEITPTVFLSVETDEL	RHMANGYQTVVSIANDPAAAKFLNTDLNNAFWTQQKYFTPALGYLFEYGSKFKVE											<i>Methylosinis</i> sp. PW1
SGDAVECSINLQLVGEACFTNPLIVAVTEWASANGDEMTPTVFLSIETDEL	RHMANGYQTVVSIANDEAASKYLNTDLNNAFWTQQKYFTPVLGMMFEYGS											<i>Methylomonas</i> sp. LW15
SGDAVECSINLQLVGEACFTNPLIVAVTEWASANGDEMTPTVFLSIETDEL	RHMANGYQTVVSIANDEAASKYLNTDLNNAFWTQQKYFTPVLGMMFEYGS											<i>Methylomonas</i> sp. LW13
SGDAVECSVNLQLVGEACFTNPLIVAVTEWASANGDEITPTVFLSVETDEL	RHMANGYQTVVSIANDPAAAKYLNTDLNNAFWTQQKYFTPALGYLFEYGS											<i>Methylosinus</i> sp. LW8
SGDAVECSINLQLVGEACFTNPLIVAVTEWASANGDEITPTVFLSVETDEL	RHMANGYQTVVSIANDPAAAKYLNTDLNNAFWTQQKYFTPALGYLFEYGS											<i>Methylosinus</i> sp. LW4
SGDAVECSVNLQLVGEACFTNPLIVAVTEWASANGDEITPTVFLSVETDEL	RHMANGYQTVVSIANDPAAAKYLNTDLNNAFWTQQKYFTPALGYLFEYGS											<i>Methylosinus</i> sp. LW3
SGDAVECSINLQLVGEACFTNPLIVAVTEWASANGDEMTPTVFLSIETDEL	RHMANGYQTVVSIANDEAASKYLNTDLNNAFWTQQKYFTPVLGMMFEYGS											<i>Methylomonas</i> sp. KSWIII
SGDAVECSINLQLVGEACFTNPLIVAVTEWASANGDEMTPTVFLSIETDEL	RHMANGYQTVVSIANDEAASKYLNTDLNNAFWTQQKYFTPVLGMMFEYGS											<i>Methylomonas</i> sp. KSPIII
PVDAVECSLNLQLVGEASVTNSLIAKGTGWAAANGDEITPTVFLSIETDEL	RHMANGYQTVVSIANDPASAKYLNTDLNNAFWTQQKYFTPVLGMLFEYGS											bacterioneuston clone
SGDAVECSLNLQLVGEACFTNPLIVAVTEWAAANGDEITPTVFLSIETDEL	RHMANGYQTVVSIANDPASAKYLNTDLNNAFWTQQKYFTPVLGMLFEYGS											bacterioneuston clone
SGDAVECSVNLQLVGEACFTNPLIVAVTEWASANGDEITPTVFLSVETDEL	RHMANGYQTVVSIANDPASAKFLNTDLNNAFWTQQKYFTPVLGYLFEYGS											bacterioneuston clone
SGDAVECSVNLQLVGEACFTNPLIVAVTEWASANGDEITPTVFLSVETDEL	RHMANGYQTVVSIANDPASAKFLNTDLNNAFWTQQKYFTPVLGYLFEYGS											bacterioneuston clone
SGDAVECSVNLQLVGEACFTNPLIVAVTEWASANGDEITPTVFLSVETDEL	RHMANGYQTVVSIANDPASAKFLNTDLNNAFWTQQKYFTPVLGYLFEYGS											bacterioneuston clone
SGDAVECSVNLQLVGEACFTNPLIVAVTEWASANGDEITPTVFLSVETDEL	RHMANGYQTVVSIANDPASAKFLNTDLNNAFWTQQKYFTPVLGYLFEYGS											bacterioneuston clone
SFDAVERSVNLQLVGEACFTNPLIVAVTEWASANGDEITPTVFLSVETDEL	RHMANGYQTVVSIANDPASAKFLNTDLNNAFWTQQKYFTPRLGYLFEYGS											pelagic clone
SGDAVECSLNLQLVGEACFTNPLIVAVTEWAAANGDEITPTVFLSIETDEL	RHMANGYQTVVSIANDPASAKYLNTDLNNAFWTQQKYFTPVLGMLFEYGS											<i>Mc. capsulatus</i>
SGDAVECSLNLQLVGEACFTNPLIVAVTEWAAANGDEITPTVFLSIETDEL	RHMANGYQTVVSIANDPASAKYLNTDLNNAFWTQQKYFTPVLGMLFEYGS											<i>Mb. capsulatus</i>
SGDAVECSVNLQLVGEACFTNPLIVAVTEWASANGDEITPTVFLSVETDEL	RHMANGYQTVVSIANDPASAKFLNTDLNNAFWTQQKYFTPVLGYLFEYGS											<i>M. trichosporium</i>
SGDAVECSVNLQLVGEACFTNPLIVAVTEWASANGDEITPTVFLSVETDEL	RHMANGYQTVVSIANDPAAAKYLNTDLNNAFWTQQKYFTPALGYLFEYGS											<i>Methylocystis</i> sp. M
SGDAVECSVNLQLVGEACFTNPLIVAVTEWASANGDEITPTVFLSVETDEL	RHMANGYQTVVSIANDPAAAKYLNTDLNNAFWTQQKYFTPALGYLFEYGS											<i>Methylocystis</i> sp. W114
SGDAVECSINLQLVGEACFTNPLIVAITEWAAANGDEITPTVFLSIETDEL	RHMANGYQTVVSIANDEAASKYLNTDLNNAFWTQQKYFTPVLGYLFEYGS											<i>M. pelagicum</i> NI

Figure 5.4. Alignments of derived amino acid sequences of a region of the *mmoX* genes from various known strains and DNA isolated from the pelagic and bacterioneuston environments. Residues in the filled boxes are universally conserved, and residues in the red box are Fe binding sites. Amino acids are numbered according to the published sequences for *Methylococcus capsulatus* (Bath) (Smith & Dalton, 1989). Mc: *Methylococcus*, Mb: *Methylobacter*, M: *Methylomicrobium*.

The sequence of amino acids produced from the analysis is extremely conserved, insufficient sequence variation for phylogenetic interpretation. This limits the conclusions we can draw from the results and shows only the presence of the target gene. This was most probably due to the high degree of conservation in the sequence between species (79.5% for MmoX protein sequence, McDonald *et al.*, 1997).

The derived amino acid sequence of the α -subunit of protein A shows one of the iron-binding domains, containing specific aspartate, glutamate and histidine residues (McDonald *et al.*, 1997), being highly conserved throughout all the sequences.

5.3.3. Attempts to grow extant marine methanotrophs

Seven marine methanotroph strains were donated to this study, for molecular analysis and for the work carried out in Chapter 4.

Three strains NI, KO, and YO given by Hiroyuki Fuse (Chugoku National Industrial Research Institute, Hiroshima, Japan) were isolated by Fuse and co-workers during their study of marine methanotrophs containing soluble methane monooxygenase (Fuse *et al.*, 1998). All three are non-pigmented, short, motile rods and only strain NI was identified to contain sMMO. Strain NI was isolated from marine mud, Hiroshima, strain KO from seawater, Hiroshima, and strain YO from seawater, Kagawa, Japan. Although strain NI was preliminary identified as *Methylobacterium pelagicum*, the other two strains were not investigated further by Fuse *et al.*, once they did not demonstrate the ability to produce sMMO.

Namba Kenji [Department of Aquatic Biosciences, University of Tokyo, Japan] donated four strains, strains O-4, O-12, 9232 and 07061. Based on

microscopic morphology, strains 9232 and 07061 were identified as *Methylococcus* species, and strains O-12 and O-4 were identified as *Methylobacter marinus*.

Growth of seven strains was achieved in Basal sea medium or IM-310 medium (section 2.5.4) using methane as the sole carbon and energy source.

However, growth of all the strains in broth was contaminated.

Molecular investigation of the cultures that were originally received also showed contamination. Samples of these original stocks were investigated using PCR primers for both *pmoA* and the universal 16S rRNA gene, the subsequent sequences obtained from the 16S rRNA analysis showed more than one organism to be present, and the sequences obtained from the *pmoA* analysis although only one sequence type detected could not be linked to those identified by the analysis. Growth on solid media and sloppy agars did not yield any single morphological colony type. Any presumptive single colony taken and subsequently grown in liquid medium also resulted in more than one morphological type, and if molecular methods were employed more than one RFLP indicating the presence of more than one type of organism.

Communication with both the laboratories from which the stocks were sent confirmed that all seven of the stocks were contaminated with unknown organisms.

5.3.4. DGGE analysis of the bacterioneuston

PCR amplification of the *pmoA* gene using the primers mb661 and A189 gc clamp (Table 2.2) to give fragments of DNA which could be separated using DGGE apparatus failed. The reasons for this are not clear but a number of different attempts were made but all failed.

5.4. Discussion

When considering oxidation and (potential) hydrosphere-atmosphere exchange, it becomes obvious that microbial oxidation plays an important role in the global CH₄ cycle (Joye *et al.*, 1999). Therefore, methanotrophic bacteria are important environmentally due to their role in carbon cycling, as well as their use in bioremediation strategies. In order to more fully apply molecular techniques associated with these important bacteria, more information regarding diversity of *in situ* populations in new environments is needed. Molecular tools are especially important as many methanotrophs are difficult to isolate on solid media, which makes growth based assessment of natural populations problematic (Costello and Lidstrom, 1999). The ability to rapidly assess and monitor natural populations of methanotrophs by using molecular techniques holds great promise for understanding the complex role of these bacteria in nature.

The control of aerobic methane oxidation is obviously related to the requirement for oxygen and methane. As a consequence, maximum oxidation rates are found where diffusion of oxygen from above and methane from below is optimal for methanotrophs (Van Der Nat *et al.*, 1997). The aim of this part of the study was to determine if this might be due to the bacterioneuston.

As has been stated earlier there is little published information on soluble methane monooxygenase in Type I methanotrophs. The results from the analysis, of this study, to identify methanotrophs in the bacterioneuston using *pmoA* as the biomarker gene show only the presence of Type I methanotrophs, whereas the experiments using *mmoX* as the biomarker may indicate the presence of Type II methanotrophs within the environment. Fuse *et al.*, (1998) have shown that *Methylobaculum pelagicum* NI, which is a Type I methanotroph, contained *mmoX*

and was able to degrade trichloroethylene in the presence of methane at the higher oxidation rates associated with sMMO (rather than pMMO). The species was isolated from the marine environment.

This would lend credence to the possibility of Type I methanotrophs containing sMMO being present in the bacterioneuston and the pelagic water. This would not be unexpected as the bacterioneuston is an “extreme” environment and any advantage an organism could gain to increase its ability to survive would be a boon. This implication may also be the case for the *pmoA* sequences found which have a high degree of identity to that sequence found in the methanotroph strain HB which inhabits another extreme environment, hot springs. This configuration of the PmoA protein conferred by this specific amino acid sequence type may well be more resistant to denaturation than other configurations and this could be useful in an environment subject to many extreme conditions. The fact that this sequence was found only in the pelagic sample might indicate that it is not present in the bacterioneuston, not enough samples were tested within the confines of this study, PCR bias, or less abundant overall. As only 2% of the pelagic library was identified to be similar to this sequence more investigation to find these sequences in the bacterioneuston could use HB specific primers. Another reason may be that in “ideal” conditions organisms with this protein could well be succeeded by those with the better known version and the sample taken from the bacterioneuston was in conditions conducive to the growth of organisms containing the more common protein.

No methanotroph-like 16S rDNA sequences were found in the bacterioneuston (see Chapter 3) but this has been experienced before. For example,

Costello and Lidstrom (1999) found two *pmoA* sequences similar to *Methylococcus pmoA* and yet they did not detect any *Methylococcus* 16S rDNA in Lake sediments.

Detailed physiological and biochemical studies were not undertaken for this study as no pure cultures of methanotrophs were isolated from the bacterioneuston or the pelagic waters sampled. Those extant marine methanotrophs donated could not be isolated to pure cultures and so again we were unable to carry out detailed physiological and biochemical studies on any marine methanotroph.

There is little published information on the properties of sMMO in Type I methanotrophs. The possession of sMMO protein has already been demonstrated in two strains, *Methylobacter methanica* strain 68-1 and *Methylobacter* sp strain GYJ3 (Koh *et al.*, 1993, Shen *et al.*, 1997). Fuse *et al.* (1998), reported the partial sequence of *mmoX* from the Type I methanotroph *Methylobacterium pelagicum* NI, however the complete DNA sequence of sMMO gene cluster from Type I methanotrophs has not been reported.

These results show only the presence of this section of the gene not the ability of the organisms within these two communities to produce soluble methane monooxygenase. Along with the results from Fuse *et al.* (1998), which demonstrated a marine *Methylobacterium* species with the ability to produce soluble methane monooxygenase, we can infer the presence of organisms that might be able to express and utilise soluble methane monooxygenase in the marine environment.

All seven strains when grown in broth congregate and form clumps, these clumps seem to be bound together by extracellular products, when these were then spread on to plates the clumps did not separate and so made it extremely difficult to separate contaminating organisms. Vortexing and serial dilutions to separate the clumps and contaminating organisms did not work, this may be as the vortexing

stressed the organisms too much and so made them unculturable or it may be that the contaminating organism and the methanotroph have formed a symbiotic relationship. Many methods, such as the use of different media, were tried to isolate the methanotrophs from these mixtures but none were successful.

Chapter Six

Discussion

6.1. Molecular investigation of the bacterioneuston vs. pelagic bacteria

In this study a comparison was made between two separate marine environmental samples. One environmental sample was from the sea surface microlayer, in particular the top 30µm of the sea, the other was pelagic water collected from about 1m below the surface. The physical differences between these environments have been covered throughout this thesis, this study concentrated on the differences between the bacterial populations of the two environments using molecular techniques to investigate. The resultant molecular analysis revealed the assemblage of the bacterioneuston to be distinct from that of the pelagic waters, in the richness of the community, that is the number of taxa present, and in bacterial abundance, i.e. the prevalence of *Vibrio* species and *Pseudoalteromonas*, within the assemblage of the bacterioneuston sampled.

The sea surface microlayer is a physical interface (as described in Chapter 1) at which organic molecules selectively partition (Hardy, 1982). Cells also occupy this environment in increased numbers as evidenced by the literature (Carty and Colwell, 1975; Kjelleberg and Hakansson, 1977; Hermansson and Dahlback, 1983; Hardy and Apts, 1984; De Souza, 1984). *Vibrio*, *Aeromonas* and *Pseudomonas* species have been regularly isolated from this sea surface (Fuhrman *et al.*, 1993; Glöckner *et al.*, 1996; Glöckner *et al.*, 1999; Eilers *et al.*, 2000).

The bacterioneuston is the biological assemblage at the air water interface and so the question posed in this study is “Does the bacterioneuston constitute a distinct community from the bacterioplankton of the pelagic water ~1m below the surface?” The community boundaries are physical and defined by the method of sampling. So the question becomes “does the bacterial assemblage located in the

space sampled by filter membranes differ in structure from the assemblage in the water column?"

Differences in community structure include:

- Richness (i.e. number of taxa)
- Abundance (i.e. relative proportion)

For these distinctions we can use the gene library but we must be wary of PCR based data. Controls for these experiments must try to negate sampling variations, such as:

- Time of day
- Season
- Weather
- Sample coverage (number of clones)
- Sample collection (membrane effect)
- DNA extraction

Due to the nature of these experiments, the time of day for sampling cannot be exactly the same for each collection, but they can be carried out over a distinct time period, such as a day, and so be controlled thus. The season and the weather are controlled by, for example, when it is possible to get out onto the North Sea and sample safely and properly. For instance, if the wind speed is too high there may be white caps or the bacterioneuston layer adhering to the sampling membrane may be blown off. Sample coverage is taken into account (using the equations in Chapter 3) and by using the same number of clones from the different environments we should be able to infer a statistical difference in species composition between the bacterioneuston and pelagic layers.

The bacterioneuston community is one which is occupying the environment

which is predominantly affected by interface processes rather than water column processes. An interface process is anything which is transferred to the water column from the atmosphere:

- Heat
- Freshwater (salinity)
- Atmospheric deposition
- Light

There is also ocean transport to the microlayer, such processes are not restricted/biased to the interface, however. Another relevant characteristic is the surface layer of hydrophobic compounds, not really a process but it is a defining characteristic.

In theory we can construct a model which will define the layers or at least indicate the boundary between the sea surface microlayer and the water column. At some point below the surface, most of the characteristics will “flip-flop” from the deep constant state to the surface variable state. This depth can be arbitrarily defined as that zone occupied by the bacterioneuston.

Holmstrom and Kjelleberg (1999) published a mini review documenting the ability of *Pseudoalteromonas* species to produce biologically active extracellular agents, which may help with the formation of a biofilm, and do show anti-bacterial, bacteriolytic, agarolytic and algacidal activities. While a wide range of inhibitory extracellular agents are produced, compounds promoting the survival of other marine organisms living in the vicinity of *Pseudoalteromonas* species have also been found. Personal communication with Holmstrom and Kjelleberg (The University of New South Wales, Sydney, Australia) revealed that they have not investigated *Pseudoalteromonas denitrificans* or any *Vibrio* species in particular. However, the

theory that *Vibrio* species, if not promoted by *Pseudoalteromonas* produced agents, are not inhibited, may be one reason why these two organisms dominate the sea surface microlayer assemblage as found in this study.

DGGE patterns from total DNA show distinct bands for bacterioneuston and bacterioplanktonic communities. RFLP patterns from total DNA show distinct variation in make up for the bacterioneuston and bacterioplankton assemblages.

The phylogenetic relationships from the pelagic assemblage are “typical” and have been found in many previous studies. The library is not unusual in relationship to previously published marine libraries (for example, Sieburth, 1971; Tsiban, 1975; Carty and Colwell, 1975; Norkrans, 1980; Conrad and Seiler, 1988; Maki, 1993).

The results of this study define a bacterioneuston as a separate environment to that of the water column which has a distinct community structure.

6.2. Gas exchange across the sea surface microlayer

The experiments in the laboratory gas exchange tank and floating gas exchange chamber yielded important information of gas exchange not previously available. The observations allowed a definition of methane as a reactive species during gas exchange in the presence of bacterial communities. Also the results indicated the presence of a community of bacteria at the interface and showed that the bacterioneuston had an effect on gas exchange across the interface.

However, these findings depend upon subtleties of the measurements in the laboratory tank that should be reiterated. As the rate of exchange over the interface for each gas had only one variable, the presence of a community of microorganisms and one variable within that community, inclusion or not of methanotrophs, the comparison between experiments is direct. Simultaneous measurements of SF₆ and

N₂O controls showed no difference between the variables. Therefore the loss of gas from the system or higher rate of removal of methane from the headspace, in the experiment containing methanotrophs and the higher concentration of methane in the headspace, indicates the presence of a group of bacteria having an effect on the methane present in the system.

As the density of methanotrophic bacteria within the natural bacterioneuston is presently unknown, rates of bacterioneuston methane oxidation are also uncertain. Nevertheless, future warming of the oceans may act to both increase surface bacterial populations and their oxidation rates.

Importantly, these experiments, by demonstrating this effect on methane, a highly stable gas, opens the possibility that other environmentally important gases may be similarly influenced. If such enhancements are found for other gases this brings into question the assumption of zero reactivity during gas exchange and future work should therefore aim to explore the possibility that it may not be specific for methane alone.

6.3. Methanotrophs in the bacterioneuston

From this study of the bacterioneuston, and the water column below methanotroph DNA was detected using PCR primers specific for genes involved in the methane oxidation pathway (Chapter 5). What is not shown in this study is whether or not these methanotrophs are active, but results from the *in situ* gas exchange tank (Chapter 4) would suggest there is methane oxidation occurring in the ocean surface microlayer. Complementing this hypothesis are those results from the experiments carried out in the closed system (Chapter 4) indicating methane oxidation by methanotrophs in the surface microlayer.

Those DNA sequences retrieved from the bacterioneuston were all related to one species of methanotroph, *Methylomicrobium pelagicum*, which has been isolated from the marine environment previously (Sieburth *et al.*, 1987; Fuse *et al.*, 1998). Fuse and co-workers (1998) identified a species of *Methylomicrobium pelagicum* strain NI which possesses part of the soluble methane monooxygenase gene cluster, they also demonstrated the presence of the enzyme soluble methane monooxygenase. The investigation into the methanotrophs present in the samples collected for this study was carried out using PCR primers specific for two different genes encoding enzymes involved in methane oxidation, soluble methane monooxygenase (MmoX) and particulate methane monooxygenase (PmoA). The *sMMO* sequences retrieved were not sufficiently varied or long enough to indicate from which species of methanotroph they originated but were adequate as a tool for detection of methanotrophs containing the gene *mmoX* from the environmental samples.

Those *pmoA* sequences retrieved from the environments were of sufficient detail to identify which methanotroph species were present within the samples. The presence of two species of methanotroph within the water column is not unexpected and it may well be possible to identify more species if a significant number of more clones are analysed. Also it must be remembered these techniques are only detecting DNA sequences of genes encoding enzymes present in methane oxidation pathways and not diagnostic for specific bacterial species.

One of the possible reasons for the detection of a methanotroph species, *Methylomicrobium pelagicum* which may contain both forms of the enzyme methane monooxygenase (particulate and soluble) is that of adaptation and survival. Any method of increased chance of survival within an environment is bound to be of use especially in an environment that can be defined as extreme.

6.4.Future work

Future work for this study would include the generation of more libraries from other sample sites including different seas, and further investigation of these libraries, as well as those already described in this study, using general eubacterial PCR primers as well as genus or species specific PCR primers to determine the bacterial composition of the bacterioneuston.

Further investigation into the assemblages of the two communities presented in this study is hampered by the number of clones needed to be analysed to give a coverage value of 99.9% (100% would require an infinite number of clones). If it is assumed that there is an equal possibility of detecting any sequence (i.e. there is no difference in abundance) then the pelagic library alone requires further analysis of at least 46,000 clones. For the bacterioneuston library, as the assemblage is predominated by two sequences, it is not feasible to calculate the specific number of clones needed to be analysed apart from to say it would be significantly higher than that of the pelagic library.

By using PCR primers specific for certain bacterial species we can increase our knowledge of the richness of the communities but not the community structure. The use of species specific DNA probes to analyse the communities would complement our knowledge of the community structure but would require the analysis of the large number of clones mentioned previously.

The use of DGGE to compare the bacterioneuston with the bacterial population of the pelagic waters illustrated the difference in community structure of the two communities.

Further work on gas exchange across the microlayer, would involve the use of the *in situ* floating gas exchange tank, with more repetitions of this study as well as investigation into gas exchange across surface microlayers around the world including those known to be in waters above gas hydrates. The tank can also be used for the analysis of the exchange of other gases. Use of the laboratory based gas exchange tank could be furthered using large volumes of seawater taken directly from the sea and analysing the removal of methane from the system. This could also be done in conjunction with radiolabelled methane to detect the methane oxidisers within the system (Radajewski *et al.*, 2000).

Continued investigation into the presence of methane oxidisers in the bacterioneuston would need to focus on the isolation of these organisms from the environment, and include the development of techniques to assess the activity of methanotrophic organisms in the environment, such as reverse transcriptase PCR and radiolabelled methane.

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